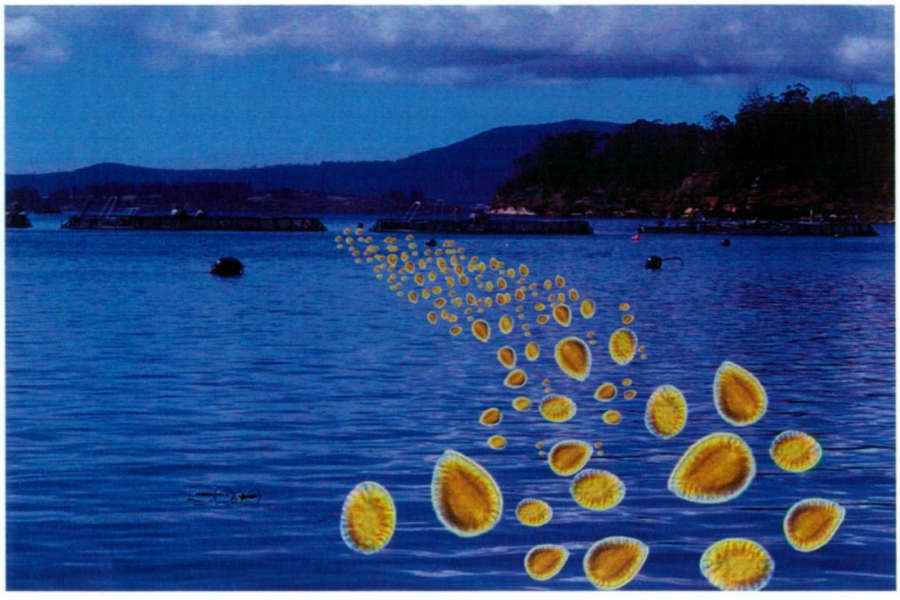
Comparative Ecophysiology, Chemotaxonomy and Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) from Australia and Japan



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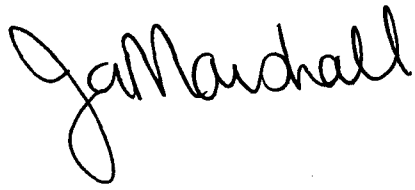

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December 2002.

Fish kill by *Chattonella* is a physiological phenomenon where diverse physiological perturbations occur both sequentially and simultaneously. Consequently, one must carefully untangle numerous intricately arrayed reactions to determine the primary physiological response to *Chattonella* exposure.

Ishimatsu 1991

Abstract

The raphidophyte flagellate *Chattonella marina* from South Australia that was associated with the mortality of farmed tuna in April 1996 was successfully cultured. This study investigates ecophenotypic variation in physiology, chemotaxonomy and ichthyotoxicity of Australian and Japanese *C. marina*.

Australian *C. marina* had similar temperature and salinity requirements to the Japanese strain but was adapted to higher light intensities than the Japanese strain. This differentiation was reflected in high concentrations of mycosporine-like amino acids (MAA's), especially the antioxidant MAA mycosporine-glycine, in the Australian cultures. Mycosporine-glycine was absent in the Japanese strain which instead used a violaxanthin:zeaxanthin cycle to moderate inhibition by high PAR irradiance.

Ecophenotypic variations in lipid profiles were also observed between *Chattonella* strains from different geographic locations. Fatty acid and sterol profiles allowed for a clear discrimination between the raphidophyte genera *Chattonella, Heterosigma, Fibrocapsa* and *Olisthodiscus*, but exhibited little differentiation between *C. marina, C. antiqua* and *C. subsalsa*. Sterol and fatty acid profiles do not support the separation of *C. antiqua* and *C. marina* as distinct species. Sterol signatures, which may be useful as chemotaxonomic markers, were identified. Lipid composition correlated more closely to recent molecular classification of raphidophytes than classification based upon carotenoid pigments.

Previous research on ichthyotoxic principles of *C. marina* has focused on production of high levels of reactive oxygen species (ROS), a brevetoxin-like compound and free fatty acids. This study found that *Chattonella marina* produces levels of the ROS superoxide 100 times higher than most algae, which is partially controlled by electrons donated through photosynthetic electron transfer. Differing superoxide production and toxic effects on zooplankton and

fish are documented between different geographic strains of *C. marina* and light treatments. These results suggest a synergistic effect between ROS and an ichthyotoxin, and cannot be explained on the basis of these mechanisms of toxicity on their own.

Our investigations into Australian *C. marina* demonstrate an absence or only very low concentrations of brevetoxin-like compounds by LC-MS techniques and negative mouse bioassays. All raphidophyte species were found to have high levels of eicosapentaenoic acid (EPA), which tested positive as a potential ichthyotoxin using damselfish as a model organism. EPA produced a similar mortality and fish behavioural response to that of intact *C. marina* cells while superoxide alone was not sufficient to cause fish mortality. However, superoxide in combination with low concentrations of EPA accelerated fish mortality. Implications of this work for mitigating the impact of *Chattonella* algal blooms on finfish aquaculture are discussed.

Acknowledgements

Gustaaf Hallegraeff who has been my boss, supervisor, colleague, friend and mentor for the past eight years and fore mostly given me a chance to further myself.

Peter Christy from PIRSA, SA who provided the sample of *C. marina* from Boston Bay in April 1996 which initiated the whole project and his constant contact keeping me in touch with the environment surrounding the tuna farming industry.

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All the students in the Harmful Algal Research Group who have passed through the lab during the years and been recruited as 'fish-observers' for my experiments, especially Yoshiko Yoshizawa, Tom Delfatti, Adrian Erberle and

Susan Lambert whom as my students played an active role in the ROS component of this work. Also Andrew Pankowski for his stimulating discussion and Miguel de Salas for company and assistance. The people of the School of Plant Science who are some of the most genuine, encouraging and inspiring people I have ever met.

Most of all I wish to thank my husband Peter Turner and children Jeffrey and Murray, who have been my sanity markers. They have kept pace with the long days, late nights, self-absorbed moods in front of the computer during this period, and kept me in touch with the rich reality of life, that there is more to life than just science.

I wish to dedicate this thesis to my brother Justin Marshall, who was a major influence in my life, encouraging me to fulfill my ambitions, but unfortunately did not stay around to see the result. Justin's blatant optimism shows through one of his encouraging statements "other peoples assessment of you are not a good measure of your own potential; only you know what you can achieve". Without this advice, I may never have taken on this project. Rest in Peace my beloved brother.

Publications arising from the thesis

This thesis is presented as an introduction (Chapter 1), a review of taxonomy (Chapter 2) and global distribution (Chapter 4), a series of 6 papers (Chapters 3, 5- 9) and concluding chapter.

Chapter 3

Marshall, J.A., P.D. Nichols and G.M. Hallegraeff, G.M., (2002). Chemotaxonomic survey of sterols and fatty acids in six marine raphidophyte algae. *J. App. Phycol.*, **14(4)**; 255-265.

Chapter 5

Marshall JA, Hallegraeff GM (1999) Comparative ecophysiology of the harmful alga *Chattonella marina* (Raphidophyceae) from South Australian and Japanese waters. *J Plankton Res* **21**:1809-1822.

Chapter 6

Marshall JA and Newman S. Differences in photoprotective pigment production between Japanese and Australian strains of *Chattonella marina* (Raphidophyceae). *J. Exp. Mar. Biol. Ecol.* **272**: 13-27.

Chapter 7

Marshall JA, Munday B, Yoshizawa Y and Hallegraeff GM (2001) Effect of irradiance on superoxide production by *Chattonella marina* (Raphidophyceae) from South Australia and Japan. . In Hallegraeff GM, Blackburn SI, Bolch CJ,Lewis RJ (eds). *Harmful Algal Blooms 2001*. Intergovernmental Oceanographic Commission of UNESCO, Paris: 316-319.

Table of Contents

Chapter 1

Chattonella marina: a potential threat to finfish aquaculture in Australia

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1.1 Introduction

The raphidophyte alga *Chattonella marina* is a small golden-brown flagellate which has two unequal flagella, possesses mucocysts and contains fucoxanthin as the principal carotenoid pigment. The cells vary in size (40-70 µm length; 20-30 µm width) and shape (ellipsoidal, tear shaped, oval or round) depending upon physiological conditions, as shown in Plate 1. The highly motile cells actively migrate through the water column, aggregating near the water surface to form an orange-brown coloration of the water, referred to as a "red-tide" or "harmful algal bloom". Harmful algal blooms (HABs) of *C. marina* are commonly associated with finfish mortalities, often within aquaculture systems. Economic losses associated with *C. marina* blooms for the finfish aquaculture industry are frequent and substantial in Japan (US$0.5 billion in 1972; Okaichi 1997). Most research on this organism has been done in Japan over the last 2 decades by 4 core research groups lead by Drs Ishimatsu, Imai, Okaichi and Onoue. Finfish mortalities have also been associated with other members of the raphidophyte family, including *C. antiqua*, *C. verruculosa* and *Heterosigma akashiwo*. However, the mode of toxicity of these raphidophytes remains unclear. Understanding the mechanism of toxicity, and how environmental factors influence toxic potential of *C. marina* is necessary for management of finfish aquaculture.

1.2 Project background

The first Australian report of a raphidophyte associated fish kill was in April 1996, when the fledgling South Australian tuna farming industry were faced with a massive mortality of their stocks of Southern bluefin tuna (*Thunnus maccoyii*). Around 7,000 tonnes of tuna worth AUS$45 million dollars died over a 3 week period (Hallegraeff *et al.* 1998). The reported cause of this fish-kill was "asphyxiation caused by the excessive mucus which covered the gills at the time" (Clarke 1996). The cause of the mucus was considered to probably be the result of injury or irritation to the gills, but the factor(s) responsible for this were not determined (Clarke 1996).

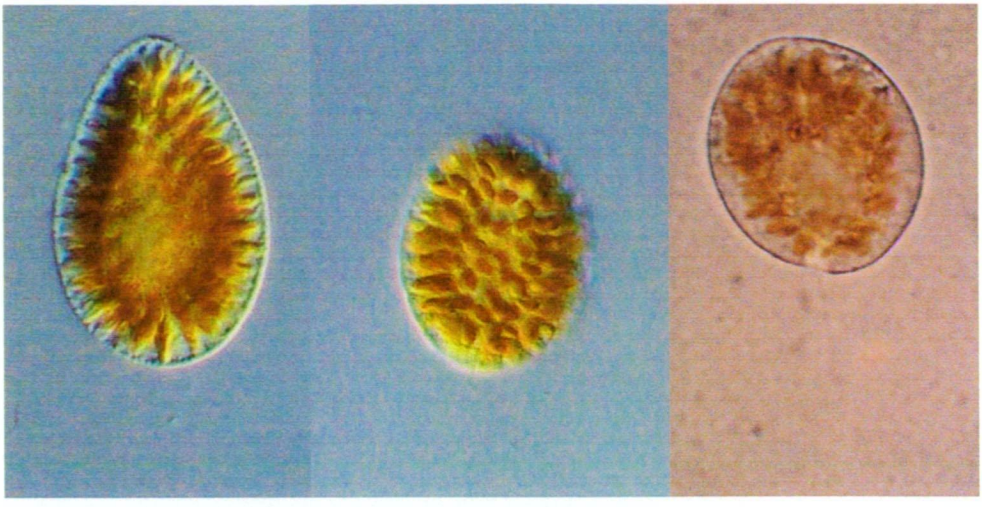

Plate 1. Different morphologies of *C. marina* (a) spindle (tear-shaped); (b) oval; (c) round.

However, analysis of water samples by Hallegraeff *et al.* (1998) revealed the presence of the ichthyotoxic raphidophyte alga *Chattonella marina* at 66,000 cells per litre in contrast to claims by Clarke (1996) that this organism was not present during the fish mortalities. Clarke (1996) also inferred that *C. marina* was not capable of blooming under South Australian conditions due to the hydrological differences between Australian and Japanese environments. In contrast Munday and Hallegraeff (1998) have concluded that the 1996 fish mortality event in South Australia may have been due to a microalgal toxicosis from *C. marina.*

1.3 Ecophenotypic differences

Thorough examination of the literature provided valuable information on global distribution and ecophysiology for all raphidophytes. Possible ecotypes were noted by analysing trends of distribution for *C. marina.* Physiological differences were difficult to ascertain due to the euryhaline and eurythermal nature of this organism, along with minimal physio-chemical data reported in conjunction with sightings. Knowledge of ecophenotypic differences between Japanese and Australian strains of *C. marina* is important for the implementation of management options for the occurrence of HABs in the vicinity of finfish

aquaculture. It has become clear that Japanese raphidophyte research cannot be readily applied to the Australian situation.

1.4 Toxicity: A complex diagnostic problem

The toxic principle of *Chattonella* (both *C. marina* and *C. antiqua*) has been widely researched since the 1970s. Initial theories for toxicity centered around toxic fatty acids, anoxia, mucus production, respiratory, ionoregulatory and cardiovascular physiology of fish exposed to *Chattonella* spp. blooms (Okaichi, 1983, Ishimatsu *et al.* 1996, Shimada *et al.* 1983, Yamaguchi *et al.* 1981, Hishida *et al.* 1998). Okaichi initially hypothesized that toxic fatty acids were responsible for finfish mortalities. Shimada *et al.* (1983) documented the production of copious quantities of mucus at the gill surface when exposed to *C. antiqua*. Matsusato and Kobayashi (1974) claimed that fish kills by *Chattonella* were due to the interception of respiratory water flow by mucus-like substance from algae. Histopathology showed severe changes in fish gill pathology when exposed to *Chattonella* spp. (Endo *et al.* 1985, Shimada *et al.* 1983, Toyoshima *et al.* 1985, Hishida *et al.* 1998).

Studies have also centred on the production of a toxin similar to brevetoxins as produced by *Gymnodinium breve* (Ahmed *et al.* 1995, Endo *et al.* 1992, Khan *et al.* 1995, Onoue *et al.* 1990). The red tide organism *Gymnodinium breve* was found to cause mass mortality of fish in Florida. Baden and Mende (1982) isolated and characterized the neurotoxic brevetoxin fractions. Further analytical studies (Onoue & Nozawa 1989, Onoue et al. 1990) have claimed that brevetoxins -like compounds or their derivatives are also produced by *C. marina*. Endo *et al.* (1988) reported that the heart rate of fish was reduced soon after exposure to *C. marina* red tide water. Later studies by Endo *et al.* (1992) reported that *Chattonella* toxins may have depressed the heart rate by depolarising the vagal nerve, causing cardiomuscular dysfunction. There were only mild alterations in the gill lamella epithelium. Endo *et al.* (1992) concluded that neurogenous cardiac disorder may be the principal cause of rapid fish kills caused by red tides of *C. marina*.

A later study by Ahmed *et al.* (1995) showed acute toxicity in *C. marina*, with two neurotoxins extracted, PbTx-2 and PbTx-3. Khan (1996) found three neurotoxic components in *C. antiqua*, CaTx-I, CaTx-II and CaTx-III which corresponded to the brevetoxin components PbTx-2, PbTx-3 and oxidised PbTx-2. In *C. marina*, Kahn *et al.* (1995) found 4 neurotoxic components. Three of them, PbTx-2, PbTx-3 and PbTx-9 have also been found in *Gymnodinium breve*. The other toxic component was an oxidised form of PbTx-2.

More recent studies has shown that many raphidophytes produce reactive oxygen species (ROS) such as superoxide ($\cdot O2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical $\cdot OH$ (Ishimatsu *et al.* 1996, Oda *et al.* 1997, Shimada *et al.* 1993, Tanaka *et al.* 1994, Yang *et al.* 1995). Links between ROS and free fatty acids from *Chattonella* have been reported by Okaichi (1983) who found that fish mortalities were caused by destruction of the gill surface by free fatty acids, an increase in lactic acid in the fish blood and suffocation due to a decrease of dissolved oxygen in the seawater. The free polyunsaturated fatty acids (PUFA) from *C. antiqua* produce superoxide (O_2^-), which cause the destruction of primary and secondary gill lamellae, represent the initial step of the fish kill. Suffocation through low dissolved oxygen and poor oxygen uptake and utilisation of oxygen are secondary causes of death.

Ishimatsu (1996) found a clear correlation between the raphidophyte cells ability to produce O_2^- and fish toxicity. The dead cells or filtrate exhibited no oxygen toxicity, which is consistent with the supposition that oxygen radicals are produced by viable cells and have a very short half life. The production of oxygen radicals is highest during the exponential growth phase (Oda *et al.* 1995) and motile spindle-shaped cells produce more radicals than round cells (Takana *et al.* 1994). Liberation of hydrogen peroxide ($H2O2$) in cellular processes can also lead to the formation of the highly reactive hydroxyl radical in a Fenton-like reaction involving superoxide and cellular Fe^{+3} (Halliwell and Gutteridge 1999).

1.5 Study rationale

The initial aim of this study was to determine if *Chattonella marina* (Australian isolate) had the ability to form blooms in conditions prevailing at the time of the fish mortality event in April 1996, Boston Bay, South Australia. Differences between the Australian strain and Japanese literature in light tolerance lead to us to access Japanese strains for comparative experimental study. The fatty acid composition of a wide range of raphidophytes strains was examined to determine inter- and intra-specific differences. Results of the chemotaxonomic and physiological studies were integrated into experiments to determine the ichthyotoxic principle.

1.6 Study outline

The result of differences in light tolerances between the Australian and Japanese strains set the groundwork for the progression of physiological 'discoveries' related to both aims of exploring the ecophysiology and toxicology of this species. The study addressed the following specific objectives;

I. Review the international (specifically Japanese) literature on *C. marina* to determine its relevance to the Australian bloom experiences (Chapters 2, 3 & 4).

II. Investigate the physio-chemical conditions of bloom formation for the Australian strain of *Chattonella marina* and comparing these with the Japanese strain (Chapter 5 & 6).

III. Determine if the differences in light tolerance between the Australian and Japanese strains affect toxicity of the species (Chapter 7).

IV. Examine the differences in toxic potential and elucidate the mechanisms of toxicity of the Australian and Japanese strain of *Chattonella marina* (Chapter 7,8 & 9).

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Chapter 2

Taxonomy of the Marine Raphidophytes

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2.1 Introduction

Raphidophytes, often referred to as chloromonads (Chloromonadophyceae in the older literature), are small 'golden-brown' fucoxanthin-containing flagellates classified in the Division Chrysophyta. Considerable confusion exists regarding the taxonomy of the group and various members have often been mistakenly assigned to other algal classes such as cryptomonads, chrysophytes, dinoflagellates and euglenoids (Taylor 1985, Hallegraeff and Hara 1995). There are a number of freshwater raphidophytes that are coloured green and have pigment affinities to the Xanthophyceae; i.e. contain diadinoxanthin, heteroxanthin and vaucheriaxanthin (or derivatives) and lack fucoxanthin and violaxanthin (Bjornland and Liaaen-Jensen 1989). The freshwater species, classified in the order Raphidomonadales Chadefaud, family Vacuolariaceae Luther, will not be covered in this work.

Members of the class Raphidophyceae were first aligned in a supragenic taxon by Klebs (1892; cited from Silva 190), Abteilung Chloromonadina of the Flagellata. This group was treated as an order Chloromonadina by Engler (1898; cited from Silva 1980), as a class Chloromonadinae by Schoenichen (1925; cited from Silva 1980) and as a division Chloromonadatae by Rothmaler (1949; cited from Silva 1980). There are many variants of the class name such as Chloromonadales (Fritsch 1927; cited from Silva 1980), Chloromonadidneae (Fritsch 1935; cited from Silva 1980), Chloromonadophyceae (Rothmaler 1951; cited from Silva 1980), as well as variants of the divisional name (Silva 1980). However, because neither the green alga *Chloromonas* Kent 1881 (cited from Silva 1980) nor *Chloromonas* Gobi 1899/1900 are members of this class, Silva (1980) has deemed the descriptive stem of Chloromonad as unacceptable.

Chadefaud in 1950 proposed the name of Raphidophyceae for the group based implicitly on *Raphidomonas*, which was later latinised to Raphidophyceae by Christensen (1962; cited from Silva 1980) replacing the name Chloromonadinae Schoenichen (Silva 1980). Silva (1980) followed the classification of Chadefaud and Christensen.

Heywood (1990) characterised the class Raphidophyceae as flagellates having the following characteristics: (i) metabolic cell lacking a cell wall; (ii) possessing two heterokont flagella, one apically directed and the other trailing; (iii) 10-20 chloroplasts each of which is surrounded by four membranes; (iv) lack of particulate storage product; (v) no eyespot; (vi) perinuclear Golgi bodies.

Loeblich and Fine (1977) recognised the similarities of the marine chloromonads in morphology and pigment type (chlorophyll *a*, c_1, c_2, and fucoxanthin) and separated *Fibrocapsa japonica*, *Heterosigma inlandica*, *Chattonella* (= *Heterosigma*) *akashiwo*, *Chattonella subsalsa* (= *Hornellia marina*), and *Olisthodiscus luteus* into the genus *Chattonella*. Many of these species were re-examined by Hara and Chihara (1982) and redefined in their separate genera. Throndsen (1993) created the Order Chattonellales which included the genus *Chattonella* Biecheler, *Heterosigma* Hada, *Olisthodiscus* Carter, *Fibrocapsa* Toriumi et Takano as well as the possibly the genus *Oltmannsia* Schiller as shown in Table 2.1

Placement of the raphidophytes in the evolutionary tree is still under debate. Gene sequencing is being used to revise the relationship of the Raphidophytes to other groups. Previously these relationships were based on light microscope and ultrastructural studies. Taylor (1985), using morphology, life cycles, biochemistry, ecology and evolutionary history as well as genetics in the determination of classification, has placed the raphidophytes close to the Dinoflagellates (Fig 2.1). Heywood (1989) questioned the placement of the raphidophytes pointing out that some carotenoids in this class showed a relationship with the Chrysophyceae where as others showed a relationship with the Xanthophyceae. However Tyrrell *et al.*(1996) found by comparing rDNA that the sequenced marine taxa from the Class Raphidophyceae (*Heterosigma akashiwo* and *Chattonella antiqua*) formed a sister grouping with the Class Eustigmatophyceae (*Nannochloropsis salina*). Potter *et al.* (1997) provides genetic evidence that the raphidophyceae has a "sister" relationship to the Xanthophyceae and Phaeophyceae.

Table 2.1. Recent taxonomic classification of the class Raphidophyceae adapted from Throndsen (1993) and Horiguchi (1996).

Division Chromophyta
(lacking chlorophyll *b*, but with chlorophyll *a* and accessory pigments)
Class Raphidophyceae Chadefaud ex Silva 1980
(many chloroplasts, anterior + posterior pointing flagellum)
Order Raphidomonadales Chadefaud
(freshwater – lacking fucoxanthin)
Family Vacuolaricaeae Throndsen

Order Chattonellales Throndsen
(marine – fucoxanthin main accessory pigment)
Family Chattonellaceae Biecheler 1936
(fucoxanthin main accessory pigment, pyrenoids)
Genera & species
Chattonella Biecheler
subsalsa Biecheler
antiqua (Hada) Ono
marina (Subrahmanyan) Hara et Chihara
globosa Hara et Chihara
minima Hara et Chihara
ovata Hara et Chihara
verruculosa Hara et Chihara
Heterosigma Hada
akashiwo (Hada) Hada ex Hara et Chihara
Fibrocapsa Toriumi et Takano
japonica Toriumi et Takano
Olisthodiscus N. Carter
luteus Carter
carterae (conspecific with *H. akashiwo*)
magnus Hulburt (possibly conspecific with *C. marina*
Oltmannsia Schiller (tentatively included)
(ribbon shaped apical flagella)
Haramonas dimorpha Horiguchi

The basic features of raphidophyte cell structure include the presence of two subequal, heterodynamic flagella arising from a more or less pronounced flagellar groove: the forward flagellum bears two rows of fine tripartite hairs, while the trailing flagellum is smooth and lies close to the surface of the cell. The cells are naked, dorsoventrally flattened and contain numerous ejectosomes (*Heterosigma)*, trichocysts and mucocysts (*Chattonella*, *Fibrocapsa*) that readily discharge (Hallegraeff and Hara 1995).

A newly discovered member of the Raphidophyte class was reported by Horiguchi (1996) and described as *Haramonas dimorpha* Horiguchi. *Haramonas*

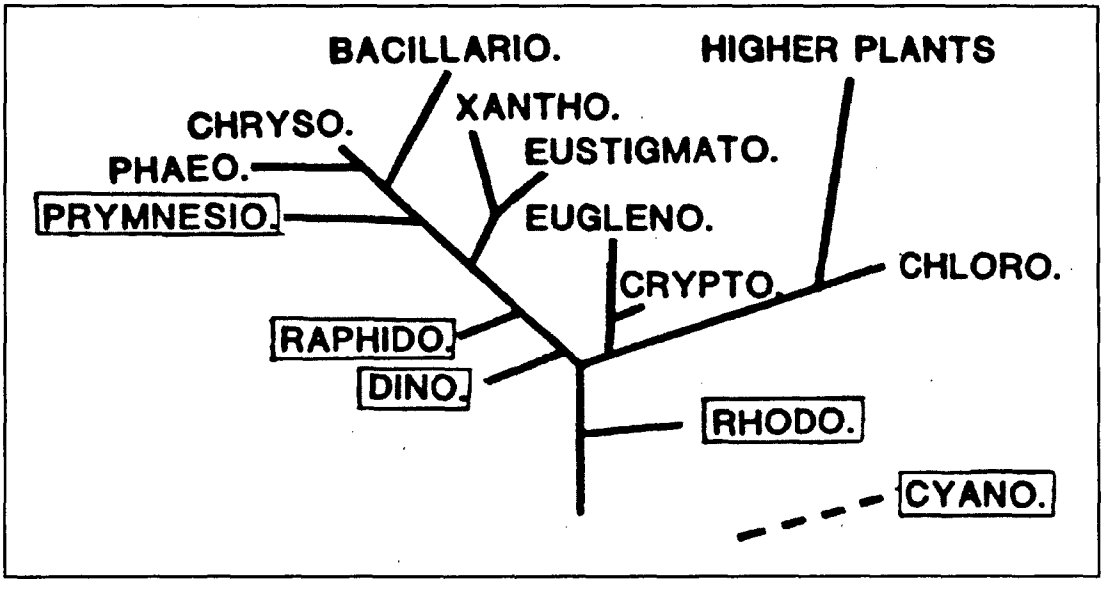


Fig 2.1. Probable relationship of the 'Lower Eukaryotes" showing the relationship of the raphidophytes to other flagellates. Adopted from Taylor (1985).

possesses raphidophycean features as described by Heywood (1990) but has unique features such as overlapping chloroplasts, a tubular invagination, and two distinct morphological stages; a club-shaped motile form and a spherical benthic non-motile form. The comparative taxonomy of the raphidophytes is detailed in Table 2.2. Notes on the taxonomic history of each species follow. Fatty acids and sterols for these species are discussed in Chapter 3.

Table 2.2. Comparative taxonomic characteristics of marine raphidophyte species. Fatty acid details are included in Chapter 3.

	Chattonella antiqua	***Chattonella marina***	***Chattonella subsalsa***	***Chattonella globosa***
Cell colour	yellow-brown	yellow-brown	yellow-brown	yellowish-brown
Cell shape	slightly flattened lanceolate slender tail	slightly flattened asymmetrical obovoid to oblong tiny protrusion from posterior end (tail) covered in tenuous mucilage	slightly flattened lanceolate	nearly globose
Measurements (μm)	50-130 long 30-50 wide	30-70 long 20-30 wide	30-50 long 15-25 wide	40-50 diam
Flagella	2 subequal heterodynamic	2 subequal equal to length of cell	2 subequal heterodynamic	2 unequal anterior longer - swimming trailing - very short
originating from	bottom of gullet	depression at anterior end of cell	bottom of anterior depression in cell	anterior depression of cell
Chloroplasts	ellipsoid arranged radially	ellipsoidal arranged radially	ellipsoidal arranged radially	discoid small, (1-2μm long,0.5μm wide)
colour		green to yellow	green to brown	pale brown to golden brown
occurring in	ectoplasm	ectoplasm	ectoplasm	ectoplasm
Pyrenoid	naked	naked in inner pole of chloroplast	in inner pole of chloroplast no thylakoids enter into pyrenoid matrix	lacking
Nucleus	tear shaped middle of endoplasm	tear shaped middle of endoplasm	tear shaped middle of endoplasm	spherical middle of endoplasm

	Chattonella antiqua	*Chattonella marina*	*Chattonella subsalsa*	*Chattonella globosa*
Contractile vacuole	absent	absent	absent	absent
Eyespots	absent	absent	absent	absent
Mucocysts	absent	absent	many oboe shaped inclusions	several large contains nail shaped inclusions along cell periphery
Pigments	Chlorophyll a, Chlorophyll $c_1 \pm c_2$, fucoxanthin (major carotenoid), ß,ß-catotene, ± zeaxanthin, ± auroxanthin, and ± violoxanthin as minor carotenoids			
Asexual reproduction	binary cell division of swimming cells	binary cell division of swimming cells	unknown	binary cell division of swimming cells
Sexual reproduction	diplontic life cycle (Yamaguchi & Imai, 1994)	diplontic life cycle anisogamous	unknown	unknown
Cyst formation	after meiosis in vegetative cell	after meiosis in vegetative cell	unknown	unknown
Cyst morphology	hemispherical simple pore at top circular wing around bottom edge	hemispherical simple pore at top circular wing around bottom edge	unknown	unknown
Distribution	South-East Asia Japan	Brackish coastal areas India Australia Japan	Eutrophic coastal Mediterranean France to Algeria	Eutrophic areas of Japan South-East Asia Canada

	Chattonella antiqua	*Chattonella marina*	*Chattonella subsalsa*	*Chattonella globosa*
Toxicity	Massive fish kills in above countries well studied	Massive fish kills in above countries well studied		known to cause respiratory damage in fish similar to other *Chattonella* sp.
Comments	*C. ovata* may be ecotype of *C. antiqua* (Fukuyo 1990, 340-431)	Often regarded as synonymous with *C. subsalsa* (Hallegraeff & Hara, 1995)	Closely related to *C. marina* but distinguished by colour and protrusions of heads of mucocysts	
	Largest *Chattonella* species	Differs from others by cell shape and size		Often confused with *C. antiqua* but differs in chloroplasts, flagellation and mucocysts

	Chattonella ovata	*Chattonella verruculosa*	*Chattonella minima*
Cell colour	yellowish-brown	pale yellow or yellow-brown	pale yellow or yellowish brown
Cell shape	fairly flattened ovoid or oblong shallow depression in anterior no depression in posterior	nearly globose several conspicuous protrusions on surface	slightly flattened, cordiform possessing shallow depression at anterior end tiny protrusion at posterior end
Measurements (μm)	50-70 long 30-45 wide	12-45 diam	20 - 45 long 20 - 30 wide
Flagella	2 subequal heterodynamic	2 unequal anterior longer - swimming trailing - very short - not beating	2 subequal heterodynamic
emerging from cell	depression at anterior	anterior	bottom of depression located at anterior end of end of cell
Chloroplasts	elongated fusiform many, arrange radially	discoid small (2-3μm long) arranged radially	ellipsoid many, arranged radially
colour occurring	between endoplasm and ectoplasm	near outer cytoplasm	in ectoplasm
Pyrenoid	naked in inner pole of chloroplast	single embedded	naked, hardly observed in inner pole of chloroplast
Nucleus	tear shaped middle of endoplasm	spherical middle of endoplasm	tear shaped middle of endoplasm
Contractile vacuole	absent	absent	absent

	Chattonella ovata	*Chattonella verruculosa*	*Chattonella minima*
Eyespots	absent	absent	absent
Mucocysts	absent	several large contains bullet-like inclusions easily ejected by physical change in environment	absent
Pigments	Chlorophyll a, Chlorophyll $c_1 \pm c_2$, fucoxanthin (major carotenoid), ß,ß-catotene, ± zeaxanthin, ± auroxanthin, and ± violoxanthin as minor carotenoids		
Asexual reproduction	binary cell division of swimming cells	binary cell division of swimming cells	binary cell division of swimming cells
Sexual reproduction	unknown	unknown	unknown
Cyst formation	unknown	unknown	unknown
Cyst morphology	unknown	unknown	unknown
Distribution	Inland sea areas of Japan	Inland sea areas of Japan	Inland sea areas of Japan
Toxicity	unknown		No record as dominant species in red tide events
Comments	Possibly conspecific with *C. antiqua* (Hallegraeff and Hara, 1995) chloroplasts.	Differs from other *Chattonella* by having verrucose appendages through cell surfaces, mucocysts and discoidal chloroplasts	Smallest Chattonella species. Distinct from *C. verruculosa* by lacking mucocysts. and from *H. akashiwo* and *F.japonica* by shape and arrangement of

	Heterosigma akashiwo	*Olisthodiscus luteus*	*Fibrocapsa japonica*	*Haramonas dimorpha*
Synonyms	*Heterosigma akashiwo*		*Chattonella japonica* (Toriumi et al. 1977) *Exuviella sp*	
Cell colour	yellow-brown to brown	yellow-green	yellowish-brown to golden brown	yellowish brown
Cell shape	slightly compressed dorso ventally ovoid to obovate 'potato-shaped' covered with tenuous mucilage oblique furrow	carapace shaped concave dorsally convex ventrally naked shallow furrow	slightly flattened ovate to obovate	variable - motile cells club shaped with slightly concave centre, rounded to sperical anterior, non-motile cells spherical tube-like invagination at posterior end 4-5μm long
Measurements (μm)	8-25 long 6-15 wide	15-25 long 10-16 wide 5-7 thick	20-30 long 15-17 wide	motile; 23-39 long 10-15 wide non-motile; 15-23 diameter
Flagella	2 subequal heterodynamic anterior -swimming posterior - rigid rotate while swimming	2 unequal anterior (1-1.2 x cell length) - beats rapidly posterior (0.8-1 x cell length) no cell rotation	2 subequal anterior - long as cell posterior - 1.2 x cell length- trailing	2 subequal heterodynamic anterior - swimming sigmoidal beating posterior -trailing
emerging from	middle of oblique furrow	the deeper well	bottom of anterior gullet	triangular anterior flagellar pit
Chloroplasts	discoid	flattened disc shaped	discoid	motile - ovoidal to discoidal
	10-30	5-13	numerous	partially overlapping, 10-20 non-motile - rod-shaped to discoidal
occurring	in cell periphery	peripheral to cell	in ectoplasm - very	arranged radially

	Heterosigma akashiwo	*Olisthodiscus luteus*	*Fibrocapsa japonica*	*Haramonas dimorpha*
			closely associated	located peripherally
Pyrenoid	protrudes from inner surface	free from thylakoids	present in each chloroplast	anterior end of chloroplast partially in cell body
Nucleus	tear shaped one or several nucleoli		centre of ectoplasm	tear shaped middle of cell
Contractile vacuole	absent	absent	absent	absent
Eyespots	absent	absent	absent	absent
Mucocysts	absent	absent	many - rod shaped - threads up to 300 µm	small in peripheral region
Asexual reproduction	binary cell division of swimming cells	binary cell division of swimming cells	binary cell division of swimming cells	unknown
Sexual reproduction	unknown	unknown	unknown multinucleate benthic stages known	unknown
Cyst formation	unknown	unknown	unknown	unknown
Cyst morphology	10 µm diam spherical, usually covered with mucilage Tomas 1978 & Ch 20	unknown	15 - 20 µm diam spherical or hemispherical if attached to diatom frustules (Matsuoka & Fukuyo 1995)	unknown

	Heterosigma akashiwo	*Olisthodiscus luteus*	*Fibrocapsa japonica*	*Haramonas dimorpha*
Distribution	estuarine and neritic Japan, New Zealand, Pacific & Atlantic, British Columbia, Chile & maybe Singapore	estuarine and neritic Japan, North America England, Europe, South Africa	coastal waters Japan, New Zealand Australia, California N. America, France	brackish waters Australia
Toxicity	causes heavy & extensive red tides from spring to autumn in Japan	unknown	has been reported a toxic effect to mariculture in Seto Inland Sea	unknown
Comments	Often confused with *O. luteus*. Distinguished by shape &colour, swimming motion, and position of flagella	Resembles *H. catenae* but green and swims smoothly	Conspicuous mucosysts & reticulate complex of chloroplast. Differs from *C. marina* in lacking tail.	

2.2 DESCRIPTION OF *CHATTONELLA*

2.2.1 Chattonella marina (Subrahmanyan) Hara et Chihara

Fig 2.2.6, 2.3.1; Plate 2.1

Basionym: *Hornellia marina* Subrahmanyan 1954. Indian J. Fish. 1, 182-203.

Illustrations: Hara & Chihara 1982 (LM, TEM), Hara, in Fukuyo.(1990) p332-333 (LM, TEM).

Hornell (1917) was the first person to describe an organism similar to *Chattonella* on the Malabar Coast of India. This organism, described as a "Euglenoid", was reported to occur in high concentration and was associated with dead fishes, crustaceans, burrowing molluscs and the sand-rooted alcyonarian, *Cavernularia*. He stated that the 'euglenoid' organism had a deleterious and fatal effect on all the other marine organisms; when living, it causes asphyxiation and when dead, liberates toxins during its decay. Subrahmanyan (1954) arrived at the 'inescapable conclusion' that the organism described by Hornell (1917), Hornell and Nayudu (1923) and Jacob and Menon (1948) under various names such as "Euglenoid", "Flagellate B", and "Protozoan", etc., and alleged to cause mortality among marine fauna were all one and the same. This was despite the lack of distinctive euglenoid feature such as the euglenoid pellicle with its numerous spiral strips.

Hara and Chihara (1982) found *Horniella marina* to be 'similar to if not identical with' *Chattonella* and transferred *H. marina* to *Chattonella*: *Chattonella marina* differs form other species of *Chattonella* by cell shape and size. The species is often regarded as synonymous with *Chattonella subsalsa* Biecheler 1936, but Hara and Chihara 1982 discuss reasons to keep them separate, i.e. no thylakoids penetrating the pyrenoids, and oboe-shaped mucocysts present in *C. subsalsa* but not in *C. marina*.

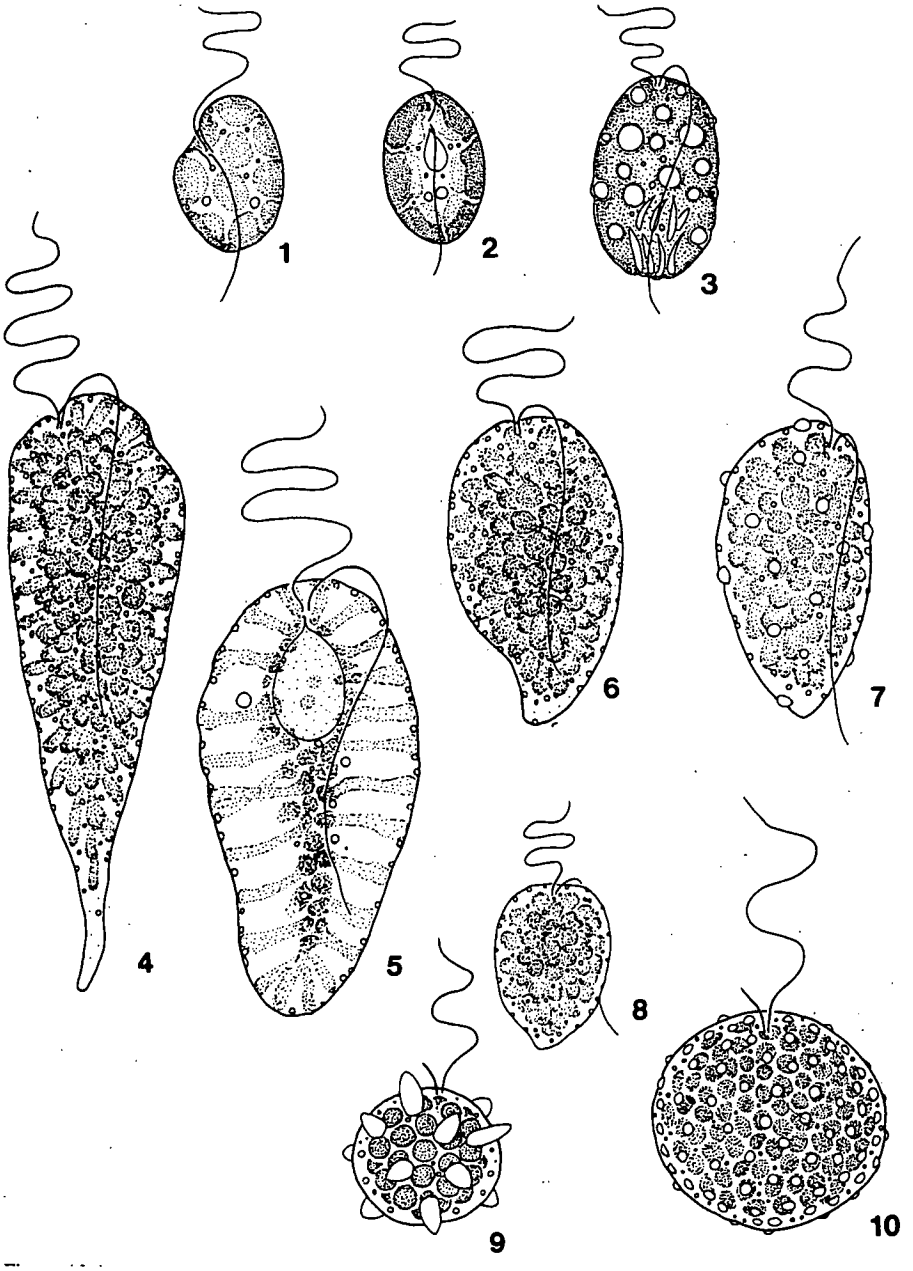


Fig. 2.2. Marine raphidophytes. Fig. 2.2.1. *Heterosigma akashiwo*; Fig. 2.2.2. *Olisthodiscus luteus*; Fig. 2.2.3. *Fibrocapsa japonica*; Fig. 2.2.4. *Chattonella antiqua*; Fig. 2.2.5. "*Chattonella ovata*" form; Fig. 2.2.6. *Chattonella marina*; Fig. 2.2.7. *Chattonella subsalsa*; Fig.2.2.8. "*Chattonella minima*" form; Fig. 2.2.9. *Chattonella verruculosa*; 2.2.10. "*Chattonella globosa*" form. Adopted from Hallegraeff and Hara (1995).

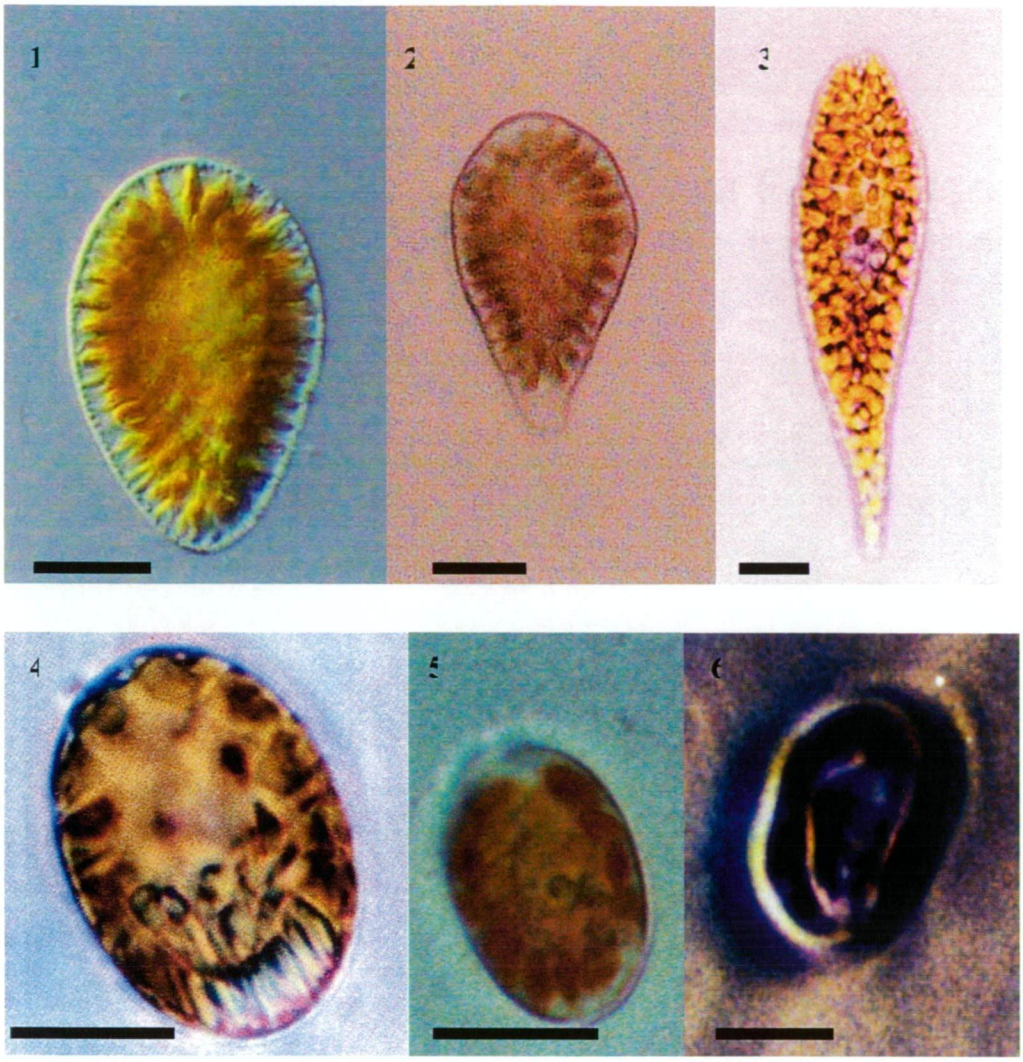


Plate 2. Raphidophytes observed under the light microscope. 2.1 *Chattonella marina*; 2.2 *Chattonella subsalsa*; 2.3 *Chattonella antiqua*; 2.4 *Fibrocapsa japonica*; 2.5 *Heterosigma akashiwo*; 2.6 *Olisthodiscus luteus*. Scale bar = 10μm

2.2.2 *Chattonella subsalsa* Biecheler

Fig 2.2.7., 2.3.2; Plate 2.2.

Illustrations: Mignot, 1976. (TEM)

Hara and Chihara (1982) separated *C. subsalsa* from *C marina* on the basis of colour and protrusion of the heads of the mucocysts on the cell surface. As it is closely related to *C. marina*, its taxonomy needs to be re-examined to reach a conclusion on the distinction between the species *C. subsalsa* and *C. marina*, but also to re-examine the validity of the delineation of the closely related genera *Heterosigma* and *Chattonella* (Hallegraeff and Hara 1995).

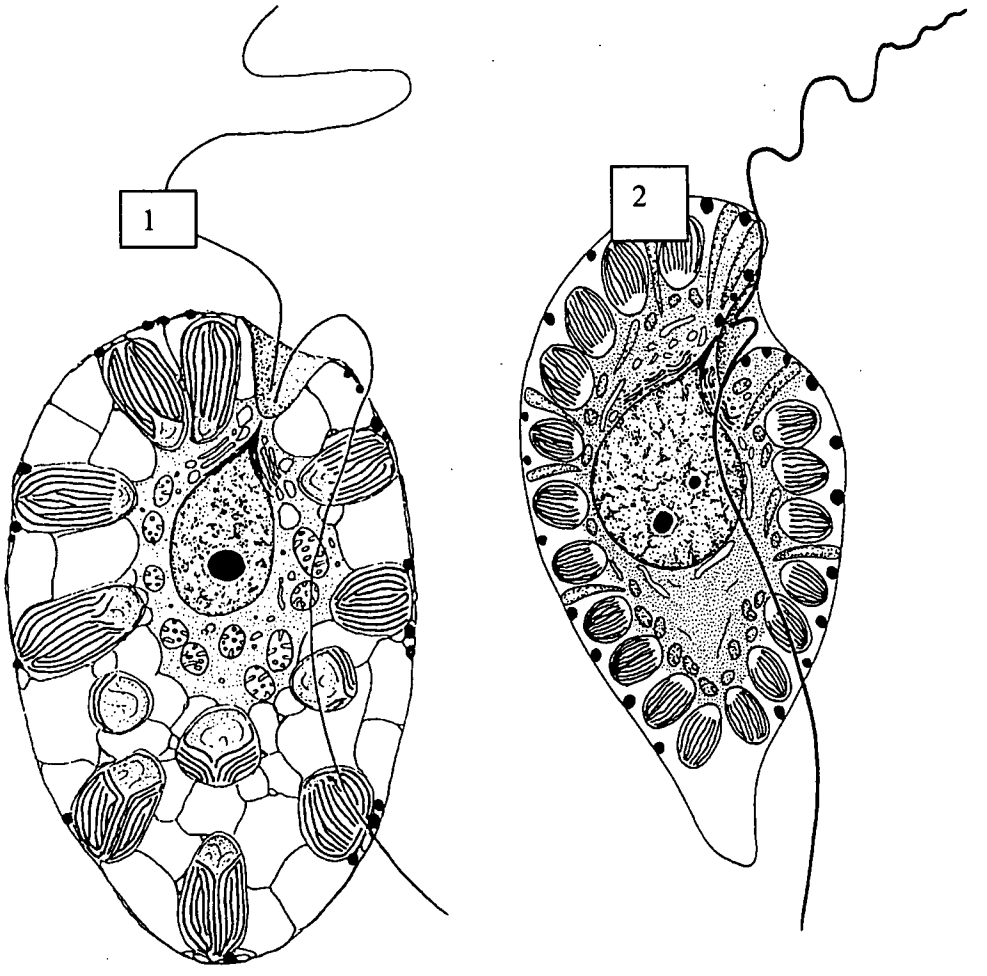
2.2.3 *Chattonella minima* Hara et Chihara (1994)

Fig 2.2.6, 2.3.3

Hara *et al.* (1994)

Illustrations; Hara, in Fukuyo (1990) p338 (LM, TEM). Hara *et al.* (1994) figs 13-18

Chattonella minima has similar flagellation and subcellular organization to *C. marina* and *C. antiqua* but has a small cell size (20-45 μm). Although similar in size to *C. verruculosa*, it lacks the large mucocysts. It has been relegated into a separate species due to its large chromosome number (>90) compared to *C. antiqua* (ca. 29) and *C. marina* (ca. 50). The form described as *C. minima* may be an ecotype of *C. marina* (Hallegraeff & Hara 1995)



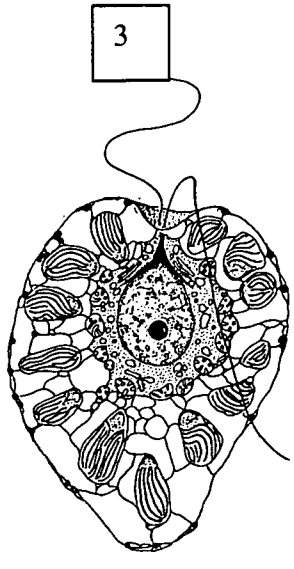


Fig 2.3. Diagrammatic illustration of *Chattonella* spp. Fig 2.3.1 *Chattonella marina* ultrastructure (adopted from Hara unpublished). Length 30-70 µm; Fig 2.3.2 *Chattonella subsalsa* ultrastructure (adopted form Mignot 1976). Length 30-50µm; Fig 2.3.3 *Chattonella minima* ultrastructure (adapted form Hara *et al.* 1994). Length 20-45µm; fa: anteriorly directed flagellum, fp:

2.2.4 ***Chattonella antiqua*** (Hada) Ono 1980

Fig 2.2.4., 2.4.1; Plate 2.3.

Basionym: *Hemieutreptia antiqua* Hada, 1974. Bull. Plankton Soc. Japan 20, 112-115

Illustrations: Hara & Chihara 1982 (LM, TEM), Hara, in Fukuyo.(1990) p332-333 (LM, TEM).

Originally described as *Hemieutreptia antiqua* by Hada (1974), it was later placed in the new class Chattonellales (Throndsen 1993) and described as *C. antiqua* by Ono and Takano (1980). This species is easily distinguished from other *Chattonella* species by its larger cell size of up to 130 μm long, however smaller morphotypes are difficult to distinguish form *C. marina* under light microscopy (see chapter 3)

2.2.5 ***Chattonella ovata*** Hara et Chihara (1994)

Fig 2.2.5, 2.4.2

Hara et al. (1994)

Illustrations; Hara, in Fukuyo *et al.* (1990) p 340 (LM,TEM). Hara et al. (1994) figs. 19-25.

Chattonella ovata differs by having well-developed vacuoles on the ectoplasm and elongated fusiform chloroplast's (Hara 1994). Imai & Itoh (1985) considered that *C. ovata* was conspecific to *C. antiqua*, but Hara et al. (1994) maintains that this taxon is two specific species characterised by a unique cell shape and the subcellular organisation related to well developed vacuolation. This species is described by Yoshimatsu & Ono (1986) as the "straw sandle-shaped *Chattonella*", and has not been reported within or outside Japan since its original description (see Chapter 3).

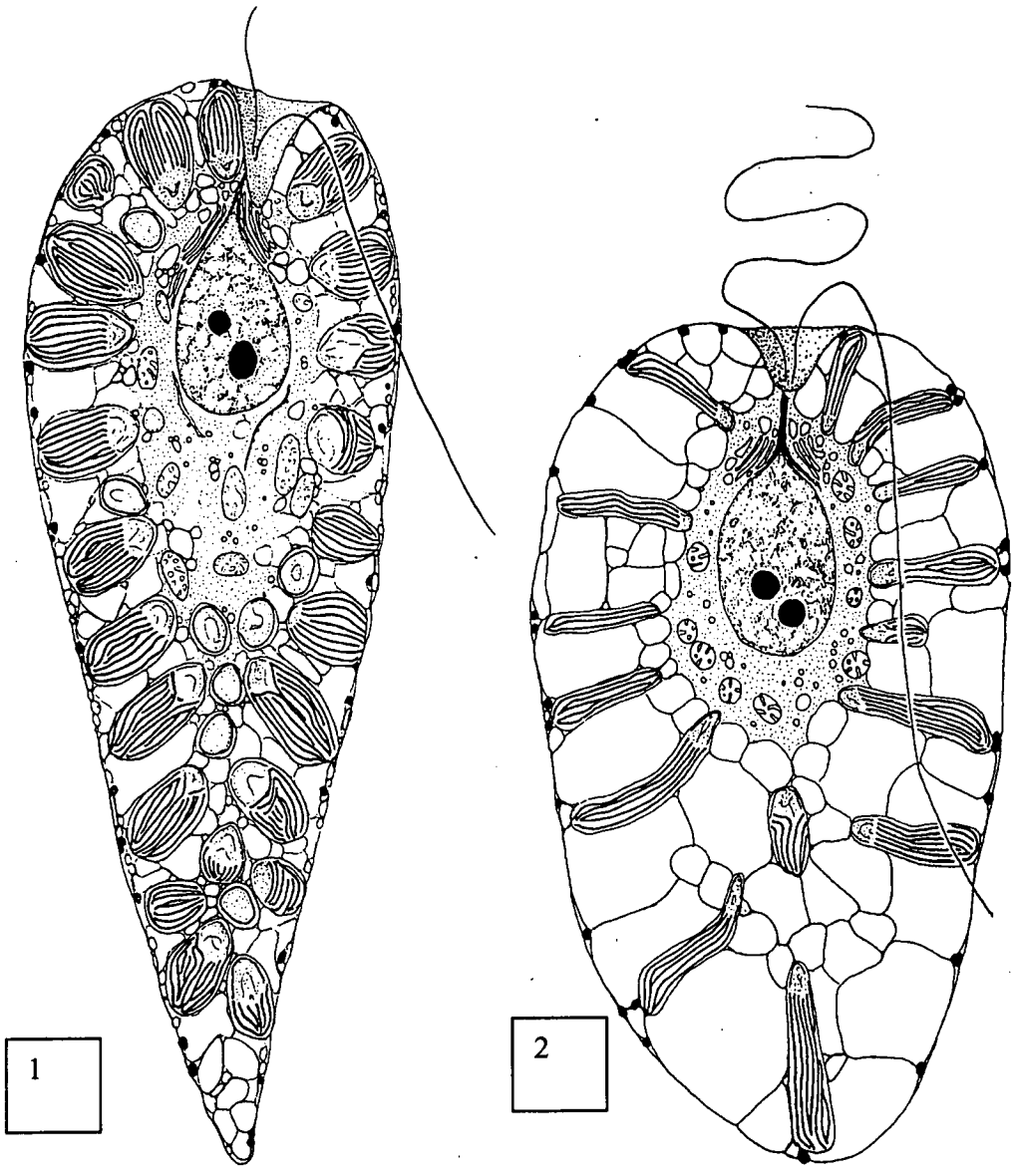


Fig. 2.4. Diagrammatic illustration of *Chattonella* spp. Fig 2.4.1 *Chattonella antiqua* ultrastructure (adopted from Hara unpublished). Length 15-130μm; Fig 2.4.2 *Chattonella ovata* ultrastructure (adopted form Hara *et al.* 1994). Length 50-70μm; fa: anteriorly directed flagellum, fp: posteriorly directed flagellum, N: nucleus, C: chloroplasts, lip: lipid globules, mu: mucocysts.

2.2.6 ***Chattonella verruculosa*** Hara et Chihara (1994)

Fig 2.2.9, 2.5.1

Nomen nudum, *in Fukuyo* et al. *(1990) p 342-343*

Illustrations; Hara, in Fukuyo *et al.* (1990) p 342 (LM,TEM). Hara et al. (1994) figs. 32-33.

C. verruculosa has verrucose appendages (or wart-like protrusions) throughout the cell surface, mucocysts with bullet shaped inclusions and discoid chloroplasts which are furnished with a single embedded pyrenoid and lacking small osmiophillic particles, which differs it from other *Chattonella* species (Hara 1994). It has no generic characters of *Chattonella* given by Biecheler (1936) and therefore may require a new genus after further investigation. Recent investigation by Sachiko (2001) has found that *C. verruculosa* has affinities with the family Dictyosa.

2.2.7 ***Chattonella globosa*** Hara et Chihara (1994)

Fig 2.2.4., 2.5.2

Nomen nudum, in Fukuyo et al., 1990. p334-335

Illustrations: Hara *et al.*, 1994. (LM, TEM); Hosaka *et al.*, 1991. (LM).

Chattonella globosa resembles *Chattonella subsalsa* by possessing mucocysts with nail shaped inclusions and by lacking genophore, contractile vacuoles and eyespots but differs from it by having spherical cell and relatively small chloroplast without pyrenoids. It has also been confused with *C. antiqua* (*Chattonella* globular type). Plasmoidal aggregations often formed under unfavourable conditions (Hara *et al.* 1994).

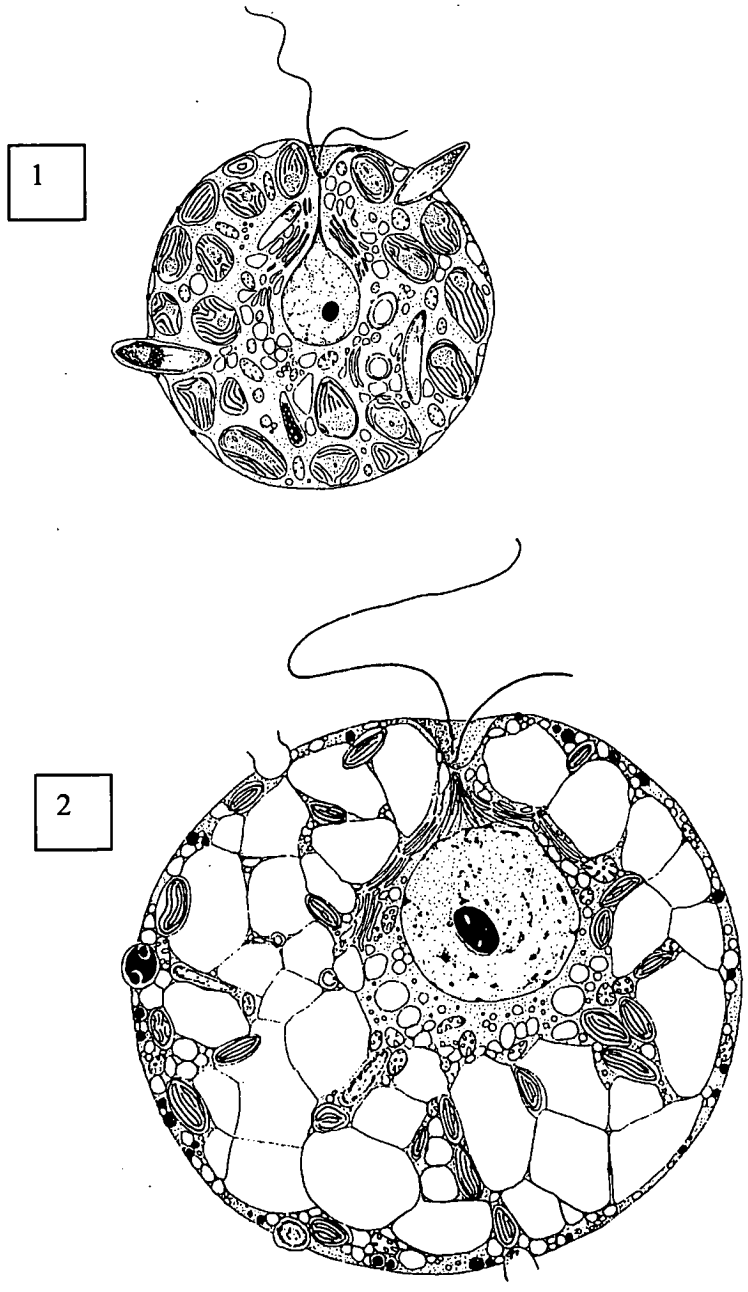


Fig. 2.5. Diagrammatic illustration of *Chattonella* spp. Fig 2.5.1 *Chattonella verruculosa* ultrastructure (adopted form Hara *et al.* 1994). Length 12-45μm; Fig 2.5.2 *Chattonella globosa* ultrastructure (adopted form Hara *et al.* 1994). Diameter 40-50μm.

Genus *Heterosigma* Hada ex Hara & Chihara, 1987. Bot. Mag. Tokyo 100: 161. Raphidophyceae, Raphidomonadales, Coelomonadaceae.

Type species: *Heterosigma akashiwo*

One marine species

2.3 TAXONOMY OF *HETEROSIGMA*

2.3.1 *Heterosigma akashiwo* (Hada) Hada ex Sournia

Fig 2.2.1, 2.6; Plate 2.4

Basionym; *Entomosigma akashiwo* Hada 1967.

Synonym: *Heterosigma inclandica* Hada 1965. J. Phycol. 1, 90

Synonym; *Olisthodiscus luteus* Leadbeater (1969), Tomas (1978), Gibbs *et al.* (1980).

Non *Olisthodiscus luteus* Carter sensu Hara.

Illustrations: Hara & Chihara 1982 (LM, TEM), Hara, in Fukuyo.*et al.* (1990) p346-347 (LM, SEM, TEM).

Heterosigma akashiwo, a common toxic red tide species in the Seto Inland Sea, Japan, has been confused with the dinoflagellates and is also known in non Japanese waters as *Olisthodiscus luteus* N. Carter. The common identity of these two was first suggested by Loeblich and Fine (1977) and, has been confirmed in detailed studies by Hara & Chihara (1985a) who found that all strains *H. akashiwo*, *H. inlandia* as well as species refered to as *Olisthodiscus luteus* were similar to one another in appearance and ultrastructural features, and could be accommodated into a single species. The error of misidentification has often been repeated when identifying blooms in European and Eastern United States waters (see Gibbs *et al.* 1980, Leadbeater 1969, Tomas 1978).

Heterosigma akashiwo can be easily distinguished from *Olisthodiscus luteus* by comparing cell shape and colour, swimming manner and emerging position of the flagella and cell shape (See Table 1. and Figs 2.2.1 & 2.2.2) .

Heterosigma exhibits a number of ultrastructural and biochemical differences which resemble the Chrysophyceae, such as the type of mitosis and carotenoid pigments, but there are also many similarities and *Heterosigma* may be looked on as a link between the two groups.(Taylor 1985).

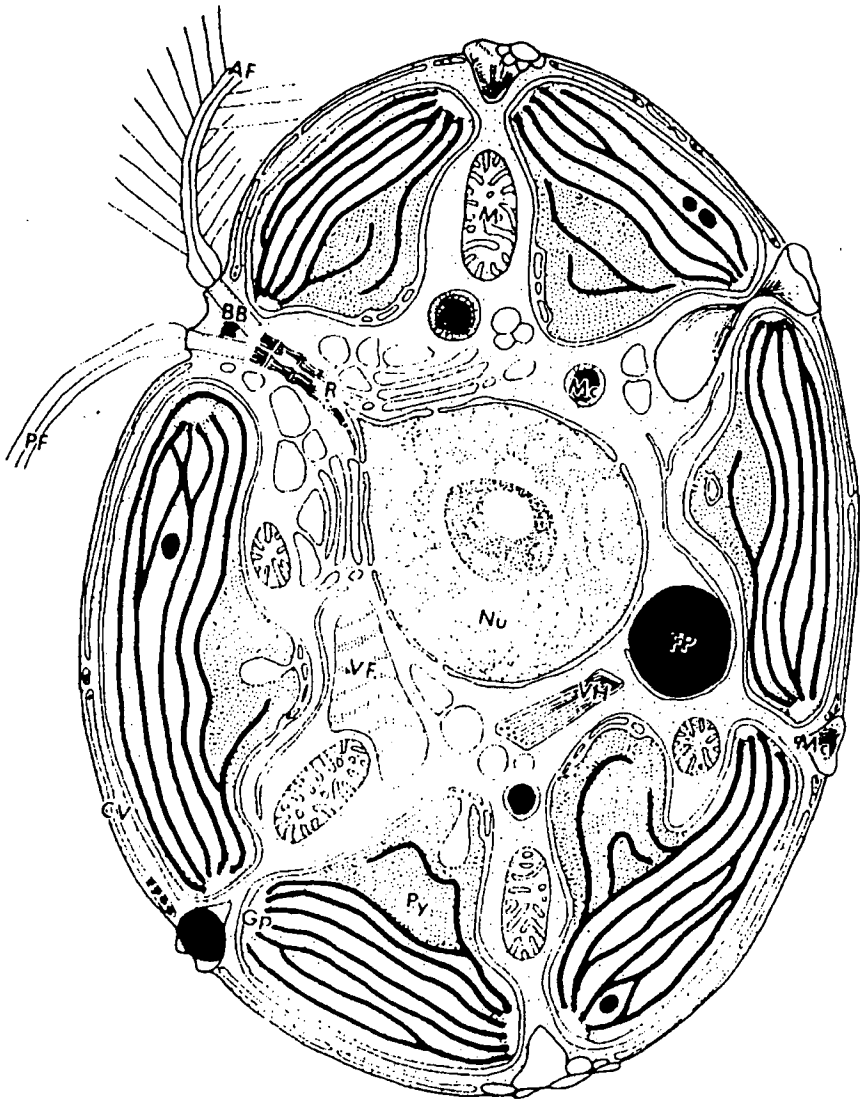


Fig. 2.6. Diagrammatic illustration of *Heterosigma akashiwo* ultrastructure (adopted form Hara & Chihara 1985a). Length 8-25μm, fa: anteriorly directed flagellum, fp: posteriorly directed flagellum, N: nucleus, C: chloroplasts, mu: mucocysts, BB: basal body, Py: pyrenoid.

In recent history, there has been confusion over the naming of *H. akashiwo*. The name change to *H. carterae* (Taylor 1992) was not accepted due to the lack of a holotype designation by Hulburt. But the holotype designated by Hara & Chihara (1987) was eventually accepted and the name changed back to *H. akashiwo* (Throndsen 1996).

Genus Fibrocapsa Toriumi & Takano, 1973. Bull. Tokai Reg. Fish. Lab. 76: 26. Raphidophyceae, Raphidomonadales, Coelomonadaceae.

One marine species

2.4 TAXONOMY OF *FIBROCAPSA*

2.4.1 *Fibrocapsa japonica* Toriumi & Takano

Figs 2.2.3, 2.7; Plate 2.5

Illustrations: Hara *et al.* 1995 (LM, TEM), Hara, in Fukuyo.*et al.*,(1990) p344-345 (LM, TEM).

Originally *Fibrocapsa japonica* was placed with the *Chattonalles* as *Chattonella japonica* by Loebich and Fine (1977) but its characteristics did not conform to those set out by Biecheler (1937) due to the lack of contractile vacuoles, possession of mucocysts as oboe-shaped inclusions, rather than rod shaped inclusions, and the large central capsule which divides the cytoplasm into the endoplasm including a large nucleus and a vacuolate ectoplasm with many chloroplasts (Hara and Chihara 1985a).

Fibrocapsa has been separated from *Chattonella* due to the prominent trichocysts instead of mucocysts (see Fig 2.3.3). At different times it has been assigned to the Chrysophyceae, Dinophyceae, and Raphidophyceae (Loeblich and Fine 1977, Nichols *et al.* 1987). Nichols *et al.* (1983) found that *Fibrocapsa* was distinct from *Chattonella* in the sterols it contains. Morphologically, *Fibrocapsa*

is also distinct from *Chattonella marina* which has a larger cell size and a tail-like protrusion at the posterior end of the cell.

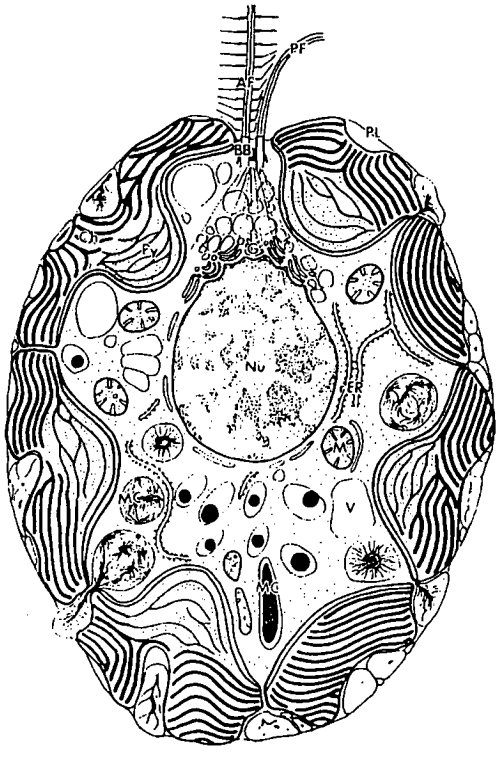


Fig. 2.7. Diagrammatic illustration of *Fibrocapsa japonica* ultrastructure (adopted form Hara & Chihara 1985a). Length 20-30μm, AF: anteriorly directed flagellum, PF: posteriorly directed flagellum, Nu: nucleus, Ch: chloroplasts, MC: mucocysts, BB: basal body, M: mitochondrium, Py: pyrenoid, V: vacuole.

Genera Olisthodiscus N.Carter, 1937. Arch. Protistenk. 90:18. Raphidophyceae, Raphidomonadales, Coelomonadaceae.

One marine species

2.5 TAXONOMY OF *OLISTHODISCUS*

2.5.1 *Olisthodiscus luteus* Carter

Figs. 2.2.2, 2.8; Plate 2.6

Illustrations: Hara *et al.* 1985b (LM, SEM,TEM); Hara, in Fukuyo.(1990) p348-349 (LM, SEM, TEM).

Olisthodiscus luteus is characterised by (1) a round or ovate cell being exceedingly flattened, and (2) a smooth gliding swimming motion instead of a rotating one as seen in *H. akashiwo*. This species has only been seen by Carter (1937) and Hara & Chihara (1982). All other recorded sightings are attributed to *H. akashiwo* (including *H. inlandica*) (Taylor 1985).

The species often referred to as *Olisthodiscus magnus* Hulburt 1965 is most likely conspecific with *C. marina*, but its description is incomplete, and a-re examination of material from the locality in Woods Hole ponds is required before a conclusion can be drawn on its precise identity.

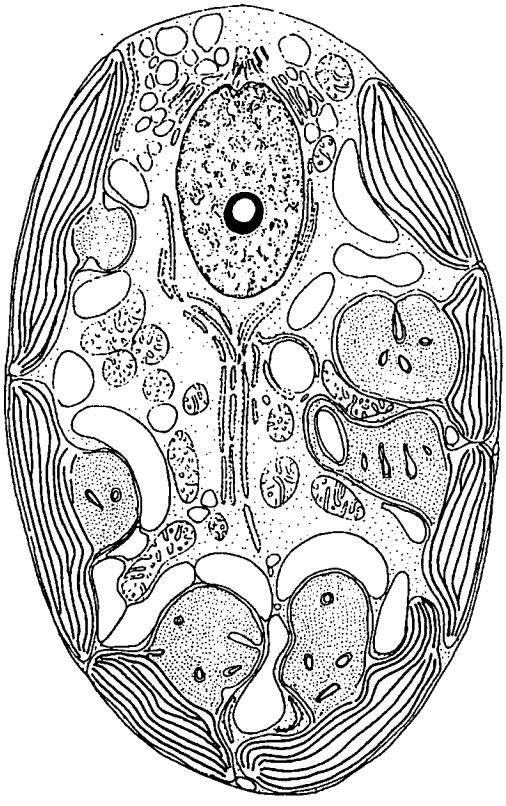


Fig. 2.8. Diagrammatic illustration of *Olisthodiscus luteus* ultrastructure (adopted form Hara and Chihara 1985b). Length 15-25µm.

Genus *Haramonas* Horiguchi, 1996. Phycological Research 44: 143-150. Raphidophyceae, Raphidomonadales, Coelomonadaceae.

One marine species

2.6 TAXONOMY OF *HARAMONAS*

Haramonas dimorpha Horiguchi

Fig 2.9

Illustrations: Horiguchi, 1996. Phycological Research 44: 143-150. (LM, TEM)

Haramonas dimorpha has two distinct phases, a club-shaped motile form, and a more or less spherical benthic non motile form. The chloroplasts in the motile form have an overlapping pattern (similar to roof tiles) with the pyreniod located at the anterior end in the chloroplast which is not overlain by another chloroplast (see Fig 2.15). The cells have a unique tubular invagination at the posterior of the cell, consisting of a single membrane supported by many flattened vesicles (Horiguchi 1996).

The spherical non-motile stage has rod-shaped or discoidal chloropasts which are arranged almost radially. Benthic cells also have a tubular invagination and possess flagella, although they are inactive. Other cellular inclusions in the benthic form are the same as those of the motile form. Benthic forms are embedded in a mucus matrix.

The species has been included in the raphidophytes by characteristics marked for Raphicophyceae by Loeblich and Fine (1977) and Heywood (1990) . Horiguchi (1996) has considered *Haramonas dimorpha* to be distinct from other raphidophytes due to the following characteristics: (i) the possession of a tubular invagination; and (ii) the unique arrangement of the chloroplasts.

There is an increasing interest in this group, as more species are likely to be discovered in the near future. A species of similar morphology was collected in Sydney in 1996 (Hallegraeff pers. comm.).

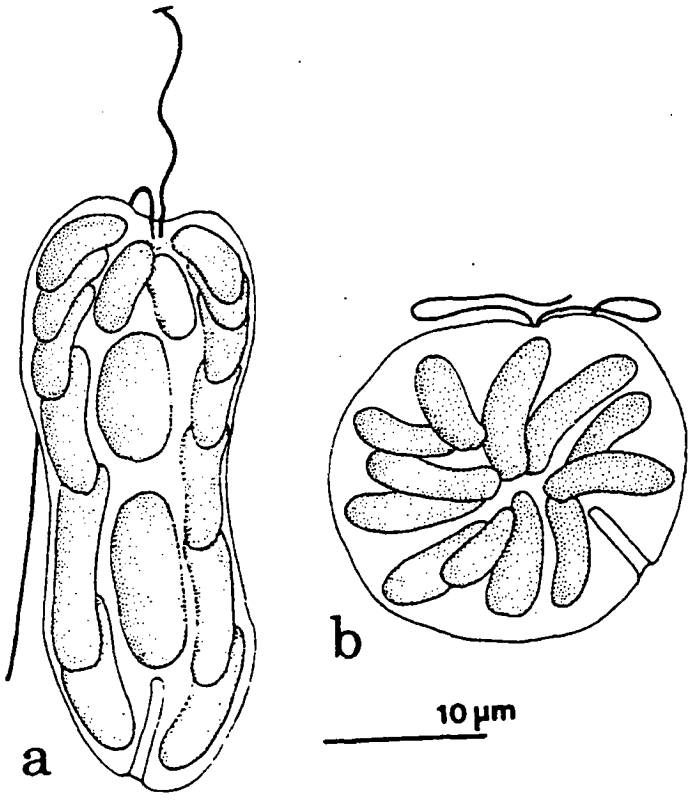


Fig. 2.9. *Haramonas dimorpha* Horiguchi showing overlapping chloroplasts and tubular invagination. a. motile cell, b. non-motile cell. Adopted from Horiguchi (1996). Length 23-39 µm.

2.7 Conclusion

There exists confusion about the taxonomy of the marine raphidophytes, especially with the *Chattonella marina* / C. *subsalsa* / C. *minima* / *C. ovata* complexes. Observations under the light microscope are insufficient to determine identity in these cases, and confusion may even exist after TEM examination. It is difficult to get quality fixation of *Chattonella* species due to their fragile nature, hence limited ultrastructural taxonomic investigation has been pursued for this species except by Hara and Chihara.

Questions over the validity of *C. minima* and *C. subsalsa* as separate species from *C. marina* have been raised. *Chattonella marina* and *C. ovata* have been shown to be indistinguishable using the ITS region or the ribosomal RNA (Connel 2000), however, *C. subsalsa* has been separated from other *Chattonella* species using the D1/D2 domain (Tyrell unpublished data). My investigation into chemo-taxonomy and distribution questions the validity of distinguishing *C. antiqua, C. subsalsa, C. minima* and *C. ovata* from *C. marina* with the limited taxonomic investigations and reported sightings available. Morphotypic variation of *C. marina* needs to be addressed prior to the description of new species. Previous chemo-taxonomic studies have reported few distinguishing features between the *Chattonella* species complexes, but have elucidated differences in fatty acid profiles of *Heterosigma, Fibrocapsa* and *Olisthodiscus* (Mostaert *et al.* 1997). The chemotaxonomy of the Raphidophytes is further discussed in Chapter 3. Further molecular research is needed to resolve these issues. The uncertainty over the identification of raphidophytes also influences the reported global distribution, as discussed in Chapter 4.

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Chapter 3

Chemotaxonomic survey of sterols and fatty acids in six marine raphidophyte algae[1]

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3.1 Abstract

Fatty acid and sterol profiles pointed clear discrimination between the raphidophyte genera *Chattonella*, *Heterosigma*, *Fibrocapsa* and *Olisthodiscus*, but exhibited little differentiation for individual *Chattonella* species *(C. marina*, *C. antiqua* and *C. subsalsa*). Sterol and fatty acid profiles do not support the separation of *Chattonella antiqua* and *C. marina* as distinct species. Ecophenotypic variations in lipid profiles are also observed between *Chattonella* strains from different geographic locations. Sterol signatures which may be useful as chemotaxonomic markers were: the absence of C_{27} sterols (cholesterol and 24-dihydrozymosterol) in *Heterosigma akashiwo*; the presence of isofucosterol in *Chattonella*; and the occurrence of brassicasterol, poriferasterol and fucosterol in *Olisthodiscus luteus*. High levels of eicosapentaenoic acid (EPA; 17-27% of fatty acids) were present in all raphidophyte species. Lipid composition correlated more closely to recent molecular classification of raphidophytes than carotenoid pigments.

3.2 Introduction

The algal class Raphidophyceae contains a number of marine species which pose a serious threat to finfish aquaculture. However, uncertainty exists over the taxonomy of this group and raphidophyte species have been misidentified as euglenoids, dinoflagellates, chrysophytes or cryptomonads. Their morphotaxonomy is mainly based on cell size and shape, and ultrastructure of chloroplasts, mucocysts, trichocysts and ejectosomes. This area of study has been pursued almost exclusively by Japanese workers (Mignot, 1976; Hara and Chihara, 1982,1985a,b, 1987; Hara *et al.*, 1994). Observations under the light microscope are often insufficient to determine species identity, especially within the *Chattonella marina / subsalsa / antiqua* complex. Confusion may persist even after examination by transmission electron microscopy as quality fixations of the fragile raphidophyte cells are difficult to achieve. Morphological features which define marine representatives of the Raphidophyceae include: naked, dorsoventrally flattened cell with two subequal, heterodynamic flagella arising from a more or less pronounced flagellar groove; the forward flagellum bears two rows of fine tripartite hairs, while the trailing flagellum is smooth and lies close to the surface of the cell. The cells contain numerous ejectosomes (*Heterosigma)*, trichocysts and mucocysts (*Chattonella, Fibrocapsa*) which are readily discharged (Hallegraeff and Hara, 1995).

Characterisation of photosynthetic pigments in unicellar algae has served to clarify disputed taxonomic affinities based on cell morphology alone. Such chemotaxonomic studies have demonstrated that raphidophytes contain a characteristic complement of chlorophylls *a*, *c1* and *c2*, and the carotenoids fucoxanthin, fucoxanthinol, *B*-carotene, zeaxanthin, violaxanthin (Bjørnland & Liaaen-Jensen, 1989; Jeffrey, 1989) and an auroxanthin-like carotenoid (Mostaert, 1998). Fucoxanthinol was reported in *Fibrocapsa japonica* and *Olisthodiscus luteus* and 19'-butanoyloxy-fucoxanthin in *Haramonas dimorpha* (Mostaert *et al.*, 1998). However, Mostaert *et al.* (1998) found that pigment composition alone did not provide the desired chemotaxonomic discrimination, but instead identified 3 pigment groups in the raphidophytes, the first consisting of *Chattonella*, *Heterosigma* and *Olisthodiscus*, the second *Fibrocapsa* and the third *Haramonas*.

Chemotaxonomic studies of lipids in raphidophytes have concentrated mainly on fatty acid composition (Bell *et al.* 1997; Cho *et al.* 1999; Mostaert *et al.* 1998; Suzuki and Matsuyama 1995; Viso and Marty 1993). Fatty acid profiles provide a more uniform and stable chemotaxonomic character for raphidophyte algae than carotenoids (Mostaert *et al.*, 1998), with significant distinctions revealed between genera. Distinguishing features are *Heterosigma akashiwo* containing 18:5, *Olisthodiscus luteus* lacking 18:4 and 22:6, but containing 20:0, and *Fibrocapsa japonica* and *Haramonas dimorpha* containing an unidentified 16:1 isomer (Nichols *et al.* 1987, Mostaert *et al.*, 1998). As with carotenoid pigments, compositional changes may occur in fatty acids due to variation in growth conditions and nutritional requirements between strains. Use of raphidophyte sterols has been largely unexplored, with only a single report for *C. antiqua* and *H. akashiwo* (Nichols *et al.* 1987). A compilation of published information on lipids of raphidophyte strains is presented in Table 3.1.

Table 3.1. Listing of strains examined in this study, together with previous literature studies of marine raphidophyte sterols and fatty acids.

Raphidophyte Species	Strain	Experimental code	Isolation details	Isolator	Sterols	Fatty acids	Reference
Chattonella antiqua (Hada) Ono	NIES-1***	CAJp	Harima-Nada, Japan	M.Watanabe 1978	+	+	Nichols et al. 1987
						+	Mostaert et al. 1998
					+	+	present work
	NIES-86		Uranouchi Bay, Japan	1980		+	Mostaert et al. 1998
	Hiroshima-71					+	Sato et al. 1988
Chattonella marina (Subrahmanyan) Hara et Chihara	NIES-14		Harima-Nada, Japan	M.Watanabe 1983		+	Mostaert et al. 1998
	NIES-121		Kagoshima Bay, Japan	T.Aramaki 1982		+	Mostaert et al. 1998
	UTCMPLO-1	CMAu#1	Pt Lincoln, Australia	J.Marshall 1996	+	+	present work
	UTCMPLO-2	CMAu#2	Pt Lincoln, Australia	J.LeRoi 1996	+	+	present work
	NIES-118#	CMJp	Seto Inland Sea, Japan	S.Yoshimatsu 1983	+	+	present work
	CWR-18*	CMNZ	Wellington Harbour, New Zealand	L.Rhodes 1999	+	+	present work
Chattonella ovata Hara et Chihara	NIES-603		Harima-Nada, Japan	I.Imai 1984		+	Mostaert et al. 1998
Chattonella subsalsa Biecheler	CCMP-217*	CSMx	Gulf of Mexico, USA	W.Gardiner 1983	+	+	present work

Raphidophyte Species	Strain	Experimental code	Isolation details	Isolator	Sterols	Fatty acids	Reference
Fibrocapsa japonica Toriumi et Takano	NIES-136***	FJJp	Kagawa, Japan	K.Yuki 1978		+	Mostaert et al. 1998
					+	+	present work
	NIES-560		Mikawa Bay, Japan	S.Toriumi		+	Mostaert et al. 1998
	CWR-02*	FJNZ	Leigh, New Zealand	L.Rhodes		+	Cho & Rhodes 1999
					+	+	present work
Misidentified dinoflagellate	FCRG-51 (lost)		San Diego, USA	J.Jordan	+	+	Nichols et al. 1983
Haramonas dimorpha Horiguchi			Daintree River, Australia	T.Horiguchi 1991		+	Mostaert et al. 1998
Heterosigma akashiwo (Hada) Hada	CS-169***	HAAu#1	West Lakes, South Australia	J.Stauber 1983	+	+	Nichols et al. 1987
	UTHAPRO1	HAAu#2	Port River, South Australia	J.Marshall 1999	+	+	present work
	CS-39=		Plymouth, England	M.Parke	+	+	Nichols et al . 1987
	MB39				+	+	present work
	CWR-05*	HANZ	Big Glory Bay, New Zealand	L.MacKenzie 1990	+	+	present work
	NIES-4		Fukuyama Bay, Japan	H.Iwasaki 1966		+	Mostaert et al. 1998
	NIES-6		Osaka Bay, Japan	M.Watanabe 1979		+	Mostaert et al. 1998
Olisthodiscus luteus Carter	NIES-15#	OLJp	Seto Inland Sea, Japan	I. Inouye		+	Mostaert et al. 1998
					+	+	present work

Strains were verified morphologically under light microscopy. *supplied by the Cawthron Institute, Nelson, New Zealand. #supplied by the National Institute for Environmental Studies, Microbial Culture Collection, Tsukuba, Japan. ***supplied by CSIRO Marine Research, Hobart, Australia. All strains used with permission from original collections.

In the present survey, we analyse both the fatty acid and sterol composition of 12 marine raphidophyte strains, and compare these with previous molecular and chemotaxonomic studies, to resolve distinguishing features between the four genera (*Chattonella*, *Heterosigma*, *Fibrocapsa* and *Olisthodiscus*) and three species of *Chattonella* (Table 3.1). Possible relationships between fatty acid composition and ichthyotoxicity of raphidophytes will also be discussed

3.3 Materials and Methods

3.3.1 *Culture conditions.*

Four strains of *Chattonella marina* originating from Australia, Japan and New Zealand, one strain of *C. antiqua* (Japan) and *C. subsalsa* (Gulf of Mexico), three strains of *Heterosigma akashiwo* (Australia, New Zealand, UK), two strains of *Fibrocapsa japonica* (Japan, New Zealand) and one strain of *Olisthodiscus luteus* (Japan) were obtained from culture collections as outlined in Table 1. Replicate strains of the Australian *C. marina* and *H. akashiwo* and replicate samples of *C. subsalsa*, *F. japonica* from New Zealand and *O. luteus* were analysed with no significant variation observed between replicates ($P > 0.05$). Cultures were grown in Ehrlenmyer flasks at 20°C in GSe medium (Blackburn *et al.*, 1988) under 150 µmol m^{-2} s^{-1} fluorescent light (Phillips daylight 18 w) on a 12:12 light:dark cycle. Culture conditions were identical for each strain. Cells were harvested in mid-exponential phase (day 7) by filtration onto pre-muffled (450°C) glass fibre filters (Whatman GF/C) and stored at -70°C until analysis.

3.3.2 Lipid extraction and fractionation

Filtered algae were quantitatively extracted using a modified Bligh and Dyer (1959) one-phase methanol/chloroform/water extraction (2:1:0.8, by volume). The samples were extracted overnight and the phases were separated the following day by the addition of chloroform and water (final solvent ratio, 1:1:0.9, by volume, methanol/chloroform/water). The total lipid was concentrated (i.e. solvents removed *in vacuo*) using rotary evaporation at 40°C.

An aliquot of the total lipid was analyzed using an Iatroscan MK V TH10 thin-layer chromatography–flame ionization detector (TLC–FID) analyzer (Tokyo, Japan) to

determine the abundance of individual lipid classes. Samples were applied in duplicate or triplicate to silica gel SIII Chromarods (5 μm particle size) using 1 μl disposable micropipettes. Chromarods were developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane/diethyl ether/acetic acid (60:17:0.2, by volume), a mobile phase resolving non-polar compounds such as wax ester (WE), triacylglycerol (TAG), free fatty acid (FFA) and sterol (ST). For selected samples, a second non-polar solvent system of hexane/diethyl ether (96:4 vol/vol) was also used to separate hydrocarbon from WE and TAG from diacylglyceryl ether (DAGE). After development, the chromarods were oven-dried and analyzed immediately to minimize adsorption of atmospheric contaminants. The FID was calibrated for each compound class [phosphatidylcholine, cholesterol, cholesteryl ester, oleic acid, squalene, wax ester (derived from fish oil) and triacyclglycerol (derived from fish oil); 0.1–10 μg range]. Peaks were quantified on an IBM compatible computer using DAPA software (Kalamunda, Western Australia). Iatroscan results are generally reproducible to ±10% or better for individual lipid classes. Either replicate strains or replicate samples were analysed for 5 of the 6 species sampled with no significant variation detected between replicate samples ($P > 0.05$).

An aliquot of the total lipid was saponified in an aqueous solution of methanolic KOH (80/20/5, MeOH/H_2O/KOH, v/v/w; 60°C, 3 h). The non-saponifiable lipids were extracted with hexane/chloroform (4:1, v/v, 3 x 1.5 mL) and treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide (50 μl, 60°C, 12 h) to convert alcohols and sterols to their corresponding O-TMSi (trimethylsilyl) ethers. After acidification of the aqueous layer, fatty acids were extracted and treated with methanol/hydrochloric acid/chloroform (10:1:1, by volume; 80°C, 2 h). Fatty acid methyl esters (FAME) were extracted into hexane/chloroform (4:1, v/v, 3 x 1.5 ml).

3.3.3 Gas chromatography and gas chromatography – mass spectrometry

Gas chromatographic (GC) analyses of non-saponifiable lipids and FAME were performed with a Hewlett Packard 5890A GC (Avondale, PA) equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m 5 0.32 mm i.d.), an FID, a split/splitless injector and an HP 7673A auto-sampler. Hydrogen was the carrier gas. Following addition of methyl nonadecanoate and methyl tricosanoate

internal standards, samples were injected in splitless mode at an oven temperature of 50°C. After 1 minute, the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C min^{-1} and finally to 300°C at 5°C min^{-1}. Fatty acid, sterol and other components were quantified with Waters Millenium software (Milford, MA, USA). GC results are subject to an error of ±5% of individual component abundance with no significant variation detected between replicate samples ($P > 0.05$). GC–mass spectrometric (GC–MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer (Austin, TX, USA) fitted with an on-column injector. Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. The GC was fitted with a capillary column similar to that described above.

3.4 Results

3.4.1 *Lipid class composition*

Thin layer chromatography-FID analysis of the total lipids of the six raphidophyte species studied (Table 3.2) indicated that lipids consisted predominantly of polar lipid (>70%, including glycolipid and phospholipid from chlorophyll and cell membrane respectively). Small amounts of sterol, free fatty acid and some triacylglycerol were also present. All strains showed similar lipid composition with the exception of the Australian isolates of *Heterosigma akashiwo* and *Chattonella marina* which had very low levels or lack triacylglycerol and contained corresponding higher levels of polar lipid. Both strains of *Fibrocapsa japonica* had high levels of free fatty acids (FFA; 23.6 - 37.9%) compared to other raphidophyte species (3.6 - 8.9%), with the exception of *C. subsalsa* which contained 18.2% FFA.

3.4.2 *Sterols*

The major sterol in the raphidophyte species studied was 24-ethylcholesterol (55.9 - 94.4%, Tables 3.3), found in very few other microalgae and seldom as a major component. Cholesterol, 2x-dihydrozymosterol and isofucosterol also occured at levels between 1.7 and 22.9 % in *Chattonella* spp., and may act as markers for this genus. In comparison to *Chattonella* spp., cholesterol was only present as a trace component in *Heterosigma*. *Olisthodiscus luteus* contained significant amounts of poriferasterol, fucosterol and brassicasterol.

Table 3.2. Relative abundances (% composition) of lipid classes for Raphidophyte species. Sample codes are designated in Table 1. n=2 duplicate samples of the same strain. # represents duplicate isolates from the same location.

	Chattonella marina				*Chattonella antiqua*	*Chattonella subsalsa*	***Heterosigma akashiwo***			***Fibrocapsa japonica***		*Olisthodiscus luteus*
	CMAu #1	CMAu #2	CMJp	CMNZ	CAJp	CSMx n=2	HAAu #1	HAAu #2	HANZ	FJJp	FJNZ n=2	OLJp n=2
Wax ester	-	-	-	-	-	-	-	-	-	-	-	-
Triglyceride	-	-	16.9	7.8	5.4	6.8	-	1.0	4.0	6.7	4.7	7.0
Free fatty acids	7.4	3.6	4.6	3.7	8.9	18.2	9.1	2.0	10.6	37.9	23.6	1.1
Sterol	1.4	1.0	0.8	0.8	1.5	1.1	1.0	0.9	2.2	1.6	1.4	1.2
Polar lipid	91.3	94.7	77.7	87.7	84.2	73.8	89.9	96.1	83.2	53.7	70.3	90.7
Total pg/cell	197.7	253.2	597.6	360.2	765.6	266.6	390.2	147.5	103.2	ND	164.5	80.9
Fatty acid/sterol	25.6	15.9	25.7	28.5	ND	22.1	26.8	36.4	29.0	ND	26.2	ND

ND = no data available. – below detection, <0.5%

Table 3.3. Sterols (% composition) for *Chattonella* species, *Heterosigma, Fibrocapsa* and *Olisthodiscus*. Sample codes are designated in Table 1. n = number of replicate samples of the same strain. # duplicate isolates from same location.

Systematic name	Common name	*C. marina*				*C. antiqua*	*C. subsalsa*	*H. akashiwo*			*F. japonica*		*O. luteus*
		CMAu #1	CMAu #2	CMJp	CMNZ	CAJp	CSMx n=2	HAAu #1	HAAu#2	HANZ	FJJp	FJNZ n=2	OLJp n=2
Cholest-5-en-3β-ol	Cholesterol	17.1	13.8	22.9	16.8	8.7	13.9	0.0+	0.0+	0.0+	11.0	5.0	36.9
Cholest-8(9)-en-3β-ol	24-Dihydrozymosterol	4.5	1.8	2.9	4.9	6.4	5.0	-	-	-	-	-	-
24-Ethylcholest-5-en-3β-ol	24-Ethylcholesterol	68.4	69.9	64.0	67.6	68.0	67.5	86.8	94.3	84.6	84.4	92.2	-
24-Ethylcholesta-5,24(28)z-dien-3β-ol	Isofucosterol	2.3	4.1	3.6	1.7	6.4	6.2	-	-	-	2.7	1.9	2.5
24-Ethylcholesta-5,24(28)E-dien-3β-ol+	Fucosterol	-	-	-	-	-	-	-	-	-	-	-	31.3
24-Ethylcholesta-5,22E-dien-3β-ol	Poriferasterol†	-	-	-	-	-	-	-	-	-	-	-	23.8
24-Methylcholesta-5,22E-dien-3β-ol	Brassicasterol†	-	-	-	-	-	-	-	-	-	-	-	0.51
Other*		7.7	10.5	6.5	9.0	10.5	7.7	13.2	5.7	15.4	1.9	0.9	5.0
Total pg/cell		6.2	9.8	16.3	8.3	23.	6.6	10.7	2.3	4.4	0.2	3.7	2.8

*other includes 24-methylene cholesterol, desmosterol, cholest-7-en-3β-ol, 24-methylcholest-5-en-3β-ol and an unknown at less than 0.5 pg per cell.
. + also contains 24-ethylcholesterol. † stereochemistry not determined. – below detection, <0.05%

3.4.3 Fatty acids

The major fatty acids found in the raphidophytes (Table 3.4) were: 16:0, 18:4ω3 and 20:5ω3. Saturated fatty acids (SFA) included low levels (7.6 – 10.7%) of 16:0 in *Fibrocapsa japonica*. High levels of 18:4ω3 were found in *Heterosigma akashiwo* (14.9-19.5%), and was a dominant component of most raphidophyte species analysed (10.8 - 26.6%). However, 18:4ω3 was only present at trace levels (0.3%) in *Olisthodiscus luteus*. All raphidophyte species contained high levels (14.8 – 24.5%) of eicosapentaenoic acid (EPA, 20:5ω3), with *Chattonella marina* containing levels of 18.5 - 23.5%. Low levels (0.4 - 1.5%) of 18:5ω3 were found in all *Chattonella* spp., and 18:5ω3 was absent in *Fibrocapsa* and *Olisthodiscus*. The monounsaturated fatty acid (MUFA) 16:1ω13t was also found in low levels in all raphidophytes.

3.5 Discussion

The raphidophyte species studied demonstrated broad similarity in the levels of lipid classes, however variations were noted within *Chattonella marina* and *Heterosigma akashiwo* in triacylglycerol levels. Triacylglycerol is a storage product and its variable production may be due to a shift in growth conditions (e.g. end of exponential phase) or may reflect ecophenotypic differences between strains. All strains were grown under identical conditions and harvested at 7 days after inoculation, suggesting that ecological differences between species from different geographical locations were the primary source of this variation. Polar lipids, including phospholipids contained in the cell membrane and glycolipid from chloroplasts, were higher in the Australian cultures of *C. marina* and *H. akashiwo*. The higher levels of free fatty acids in *F. japonica* were possibly due to its small cell size and faster growth rate leading to a greater proportion of stationary phase cells at the time of harvest. Simultaneous analysis of other biological samples, including of microbial origin, showed only low levels of FFA, therefore the high levels of FFA in several species in this study is therefore not an artefact of sampling or analytical protocol.

Table 3.4. Fatty acid composition (%) of raphidophyte species. Sample codes are designated in Table 1. n = number of replicate samples of the same strain. # = duplicate strains from the same location.

	C. marina				*C. antiqua*	***C. subsalsa***	*H. akashiwo*			***F. japonica***		*O. luteus*
	CMAu #1	CNAu #2	CMJp	CMNZ	CAJp	CSMx n=2	HAAu #1	HAAU #2	HANZ	FJJp	FJNZ n=2	OLJp n=2
Saturates												
14:0	11.9	11.8	5.4	7.4	6.8	7.7	5.9	5.8	7.8	18.5	20.1	4.6
16:0	22.3	17.1	21.2	19.8	25.8	21.0	25.3	22.8	25.6	7.6	10.7	19.4
18:0	0.8	0.8	1.6	1.6	2.2	1.9	1.2	0.6	1.5	1.6	0.7	4.4
Sum SFA	35.0	29.8	28.2	28.8	34.8	30.6	32.3	29.2	34.9	27.6	31.4	28.4
16:1ω7c	9.4	7.5	10.1	8.1	9.9	4.4	6.9	7.2	8.1	2.6	1.7	8.4
16:1ω13t	0.2	1.9	2.6	1.2	2.4	1.7	2.6	3.3	2.7	1.2	1.0	0.2
18:1ω9c	4.5	5.2	9.9	9.3	6.5	13.5	5.5	5.0	4.4	7.4	6.3	15.6
18:1ω7c	1.7	1.0	1.7	2.4	1.4	1.6	2.4	1.0	1.6	0.5	5.7	2.6
Sum MUFA	15.8	15.5	24.3	21.0	20.3	21.2	17.5	16.5	16.8	11.6	14.7	26.8
GLA 18:3ω6	0.2	0.3	0.3	0.1	0.0	0.4	0.3	0.1	0.3	1.3	0.8	0.3
18:5ω3	0.4	0.7	0.7	0.4	0.4	1.5	7.1	6.7	5.7	0.0	0.0	0.0
18:4ω3	13.7	19.0	12.7	14.9	12.5	13.2	14.9	19.5	16.5	26.6	12.0	0.3
18:2ω6	1.9	2.5	1.5	2.0	2.0	3.1	3.2	1.7	2.8	2.9	3.1	2.2
AA 20:4ω6	1.7	0.0	5.5	4.1	3.7	2.9	1.8	0.8	1.3	4.4	7.2	7.7
EPA 20:5ω3	22.9	23.4	18.5	19.8	17.7	19.5	16.9	18.7	17.1	17.4	24.5	14.8
20:4ω3	0.7	0.9	0.6	0.6	0.4	0.9	0.4	0.7	0.4	0.7	1.1	0.3
DPA 22:5ω6	2.4	2.8	1.4	1.6	2.0	0.4	0.3	0.1	0.1	0.6	0.1	3.6
			C. marina		*C. antiqua*	C. subsalsa		*H. akashiwo*			*F. japonica*	*O. luteus*

	CMAu #1	CNAu #2	CMJp	CMNZ	CAJp	CSMx n=2	HAAu #1	HAAU #2	HANZ	FJJp	FJNZ n=2	OLJp n=2
DHA 22:6ω3	3.4	3.1	3.3	3.5	2.9	3.2	3.2	3.1	2.8	0.4	0.2	3.4
22:4ω6	0.2	0.3	1.0	0.8	0.4	0.6	0.3	0.0	0.1	0.3	1.7	2.6
22:5ω3	0.2	0.4	1.0	0.8	0.4	0.6	0.8	0.3	0.5	0.0	0.0	1.7
Sum PUFA	47.8	53.3	46.4	48.7	42.3	46.3	49.2	51.7	47.4	54.5	50.7	36.6
Other* (%)	1.5	1.5	1.1	1.5	2.6	1.9	1.1	3.9	1.0	6.2	3.2	8.2
Total pg/cell	159	156	419	237	ND	165	286	84	127	25	97	ND

*other includes br 15:0, 15:0, 17:0, 20:0, 17:1, 18:1ω7t, 18:1ω5t, 20:1, 20:2, 20:3ω6, 22:1 and an unknown at less than 0.5 pg per cell. ND, not determined

Sterol compositions reported in this study were similar to those found by Nichols *et al.* (1997) for *Chattonella* and *Heterosigma*. Within *Chattonella* spp, there were no significant differences in sterol compositions (ANOVA, $p<0.001$). The presence of poriferasterol and fucosterol as well as brassicasterol in *Olisthodiscus luteus* is unique within the group. Fucosterol, a common component of brown algae, has not often been reported in marine phytoplankton. The above combination of sterols may provide new markers for these species for potential use in monitoring programmes.

The overall composition of fatty acids within the raphidophytes was uniform, although some chemotaxonomic differences were noted. Fatty acid profiles were similar to results found by others (Bell *et al.*, 1997; Mostaert *et al.*, 1998; Nichols *et al.*, 1987; Suzuki *et al.*, 1995).

For *Heterosigma akashiwo*, levels of 18:5ω3 in this study (5.7 - 7.1%, Table 4) were similar to previous reports (Bell *et al.*, 1997; Bodennec, 1998; Nichols *et al.*, 1987; Mostaert *et al.*, 1997; Viso & Marty, 1993). Low levels (0.4 - 1.5%) of 18:5ω3 were found in all *Chattonella* spp., which had not previously been reported.

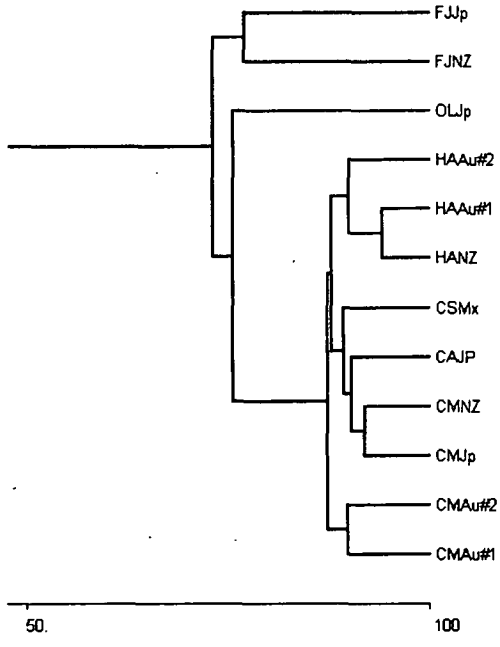

Figure 3.1. Dendogram (Bray-Curtis cluster analysis – single link) of fatty acids from raphidophyte algae showing grouping of *Chattonella marina* (CM) with *C. antiqua* (CA) and *C. subsalsa* (CS). Subgrouping of the Australian strains of *C. marina* (CMAu) are distinct from Japanese (CMJp) and New Zealand (CMNZ) strains. Sample codes designated in Table 1.

The fatty acid data, represented in a Bray-Curtis cluster analysis (Figure 3.1), demonstrated a high similarity between *C. marina*, *C. antiqua* and the strain from the Gulf of Mexico provisionally designated as *C. subsalsa*. This compares favourably with recent molecular studies by Hirashita *et al.* (2000) and Connell (2000) who found little variation in the D1/D2 domains of the ribosomal RNA and ITS regions of *C. antiqua* and *C. marina* respectively. Connell (2000) found less than 0.01% divergence using pairwise comparison between *C. marina* and *C. antiqua* suggesting that they are conspecific. Mostaert *et al.* (1998) also found no distinction between *C. marina* and *C. antiqua* fatty acids, but both differed significantly from *C. ovata* which had a much higher percentage (51.5%) of saturated fatty acids than the two former species.

Sterol data, when compared in a cluster analysis (Figure 3.2), showed that the Australian and New Zealand *Chattonella* species grouped more closely than the Japanese strains, consistent with the existence of geographical ecophenotypes. Ecophenotypic differences in ultra-violet protective pigment composition between the Japanese and Australian isolates of *C. marina* have also been previously reported (Marshall & Hallegraeff, 1999; Marshall and Newman, 2002).

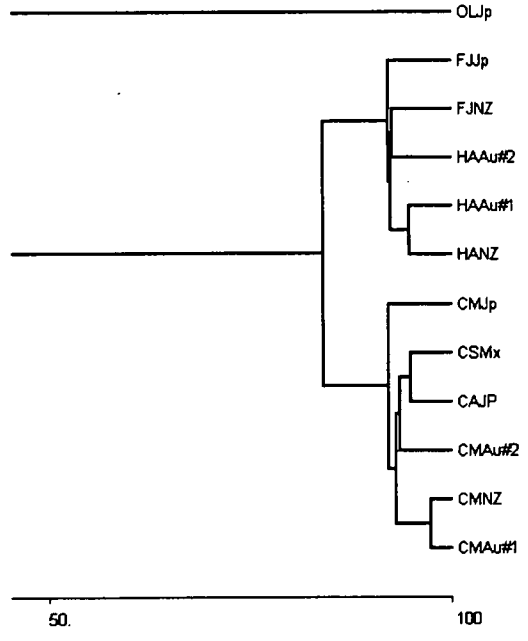

Figure 3.2. Dendogram (Bray-Curtis cluster analysis – single link) of sterols showing close grouping of raphidophyte species and mixing of *Chattonella* strains with *C. subsalsa* closely linked. The *Heterosigma* strain strain (HAAu#2) is linked to *Fibrocapsa* (FJNZ) due to similar levels of 24-ethylcholesterol. Sample codes designated in Table 3.1.

Using D1/D2 domains of the ribosomal RNA, Hirashita *et al.* (2000) could not separate *C. marina* and *C. antiqua.* Instead culture strains were discriminated according to different geographical populations within the Seto Inland Sea. Connell's (2000) results suggest that *C. subsalsa* (CCMP-217) showed a divergence of 9.8% from *C. marina* - *C. antiqua*, indicating that this strain may represent a distinct species. This suggestion is supported by our fatty acid and sterol cluster analyses. Questions over the validity of the species *Chattonella minima* and *C. subsalsa* as distinct from *C. marina* have been raised previously (Hallegraeff and Hara, 1995). *Chattonella marina, C. antiqua* and *C. ovata* have also been shown to be genetically indistinguishable using the rDNA D1/D2 domain (Sako *et al., 2000*). Hirashita *et al.* (2000) claimed that homology of the nucleotide sequences between 2 strains of *C. marina* and 4 strains of *C. antiqua* was 99.4-95.6%, which was too close to separate the two species.

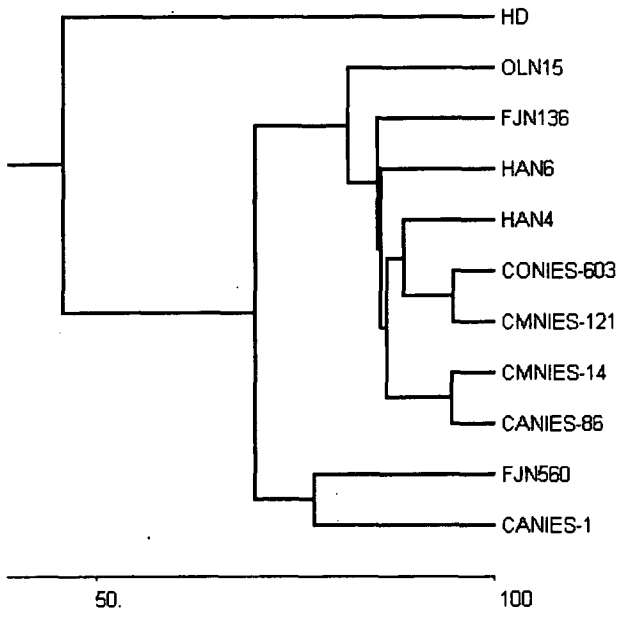

Figure 3.3. Dendogram (Bray-Curtis Cluster Analysis – single link) of carotenoid pigments in raphidophyte species using Mostaert's *et al.* (1998) data, showing mixed species grouping. Strain designations are as follows: *Chattonella antiqua* (CANIES-1*, CANIES-86); *C. marina* (CMNIES-14, CMNIES-121); *C. ovata* (CONIES-603); *Fibrocapsa japonica* (FJNIES-136, FJNIES-560); *Haramonas dimorpha* (HD); *Heterosigma akashiwo* (HANIES-4, HANIES-6); *Olisthodiscus luteus* (OLNIES-15*). * denotes the strains used in the present study.

The comparison between Bray-Curtis cluster dendograms based on pigment and fatty acid compositions and Mostaert's *et al.* (1998) carotenoid data (Fig 3.3), suggests that pigment composition may not be as reliable as fatty acid profiles for use as chemotaxonomic markers. Previous analyses of raphidophyte chemotaxonomy have occasionally revealed mistaken species identifications. The reanalysis in this study of strains of *Fibrocapsa japonica* now strongly indicates the mistaken identity of FCRG51 (Nichols *et al.*, 1983) which almost certainly was a dinoflagellate species. Similar mistaken identification of putative raphidophyte strains (as *Heterosigma akashiwo*) refers to Loeblich strain 395 which based on pigment composition was also a dinoflagellate (Fiksdahl *et al.*, 1984).

Our data sterol and fatty acid data agree with others (Conell, 2000; Mostaert *et al.*, 1998; Sako *et al.*, 2000) and we propose that *C. antiqua* and *C. marina* are conspecific. Only low numbers of *C. antiqua* have been reported outside Asian-Pacific waters and these often co-occurred with *C. marina*. Morphological variability of *C. marina* needs to be defined prior to the designation of further species, subspecies or varieties. Previous chemotaxonomic studies have shown significant differences in fatty acid profiles between *Chattonella*, *Heterosigma*, *Fibrocapsa* and *Olisthodiscus,* but also did not distinguish between *C. marina* and *C. antiqua* (Mostaert *et al.,* 1997). Hiroishi *et al.* (1989) and Vrieling *et al.* (1995) demonstrate inconsistencies in distinguishing *C.marina* from *C. antiqua* using monoclonal antibodies. The consistency of lipid data between the same Chattonella spp. Isolated between 1982 and 1999 indicates that any genetic shifts due to the age of the cultures is minimal. Ecological or geographic population differences within *Chattonella* must also be taken into consideration when developing molecular probes and monoclonal antibodies for identification.

All raphidophyte species examined in this study had consistently high levels of EPA 20:5ω3. The presence of eicosapentaenoic acid (EPA, 20:5ω3) in all raphidophyte species is of considerable interest, having been linked to toxicity in *Heterosigma* as well as in the dinoflagellate *Karenia mikimotoi* (reported as *Gymnodinium cf. nagasakiense*; Arzul *et al.*, 1995, 1998; Bodennec *et al.*, 1995, 1998). *Chattonella marina* was found to have high levels of EPA (18.5 - 23.5%) equating to 1.5 – 2 mg L^{-1} culture medium (at experimental cell density). Long chain polyunsaturated fatty acids (PUFA) with 5 or 6 double bonds are claimed to have an

allelopathic role at concentrations above 0.5 mg L^{-1}, and EPA has been shown to be the most toxic fatty acid to scallop (*Pecten maximus*) embryos and highly repressive for bioluminescence activity of *Photobacterium phosphoreum* (Arzul *et al.*, 1998). It would be of considerable interest to perform toxicity screening of raphidophyte algae in conjunction with detailed lipid class and fatty acid profiling. We suggest that the form of fatty acid may play a key role in the ichthyotoxicity of these harmful bloom forming flagellates. Okaichi (1989) reported that the highly unsaturated fatty acids 16:4 and 18:4 may be the primary causative substances in finfish mortalities. Arzul et al (2000) claimed that oxygen is a cofactor to PUFA activity, and their mode of action is probably correlated to oxygen radical formation. A synergy between neurotoxic effect and reactive oxygen species in causing finfish mortalities has been suggested for *C. marina* (Marshall *et al.*, 2001) and the relationship between fatty acids, neurotoxic action and reactive oxygen species needs to be further explored.

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Chapter 4

Global Overview of the Distribution and Ecology of Marine Raphidophyte Species

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4.1 Introduction

Knowledge of algal species distribution and ecology needs to be acquired to be able to determine bloom stimuli. Patterns of distribution may indicate physiological requirements such as temperature and salinity ranges for a particular species to bloom. Local influences such as land runoff and the associated input of terrestrially-derived dissolved organic matter (humus) have been implicated in the triggering of dinoflagellate blooms (Doblin *et al.* 1998). Anthropogenic factors may produce an excess of industrial or domestic waste, producing a chemically altered habitat (Smayda 1998), also inducing blooms. Intensive aquaculture industries, particularly finfish, may also be implicated in the stimulation of potential harmful algal blooms through the waste products of aquaculture species and the excess of food.

Temporal patterns indicate necessary seasonal or meteorological prerequisites for blooms; for example, the dinoflagellate *Gymnodinium catenatum* requires periods of calm weather after heavy rain to allow blooms to occur (Hallegraeff 1995). An algal bloom may be influenced by global climatic conditions, and cyclic events such as El Niño (Southern Oscillation) producing upwelling of nutrient rich water or changes in water column stratification (Smayda 1998, Rhodes *et al.* 1993).

Raphidophytes lack a rigid cell wall and tend to change their size and shape rapidly according to environmental conditions (Hiroshi *et al.* 1988), as well as during microscopic observations, often leading to mis-identification. For example *H. akashiwo* has often been erroneously reported in studies of Northern American coastal waters as the sand-dwelling species *Olisthodiscus luteus* (Taylor and Haigh 1993). Similarly, *Chattonella antiqua*, *C. marina*, *C. minima* and *C. subsalsa*, with very similar morphology, are easily confused (Chapters 2 & 3). Sampling of raphidophytes is also difficult due to the lack of a rigid cell wall as the cells lyse easily with unsuitable preservation techniques, can extrude through fine plankton net meshes and therefore are often missed in routine phytoplankton monitoring.

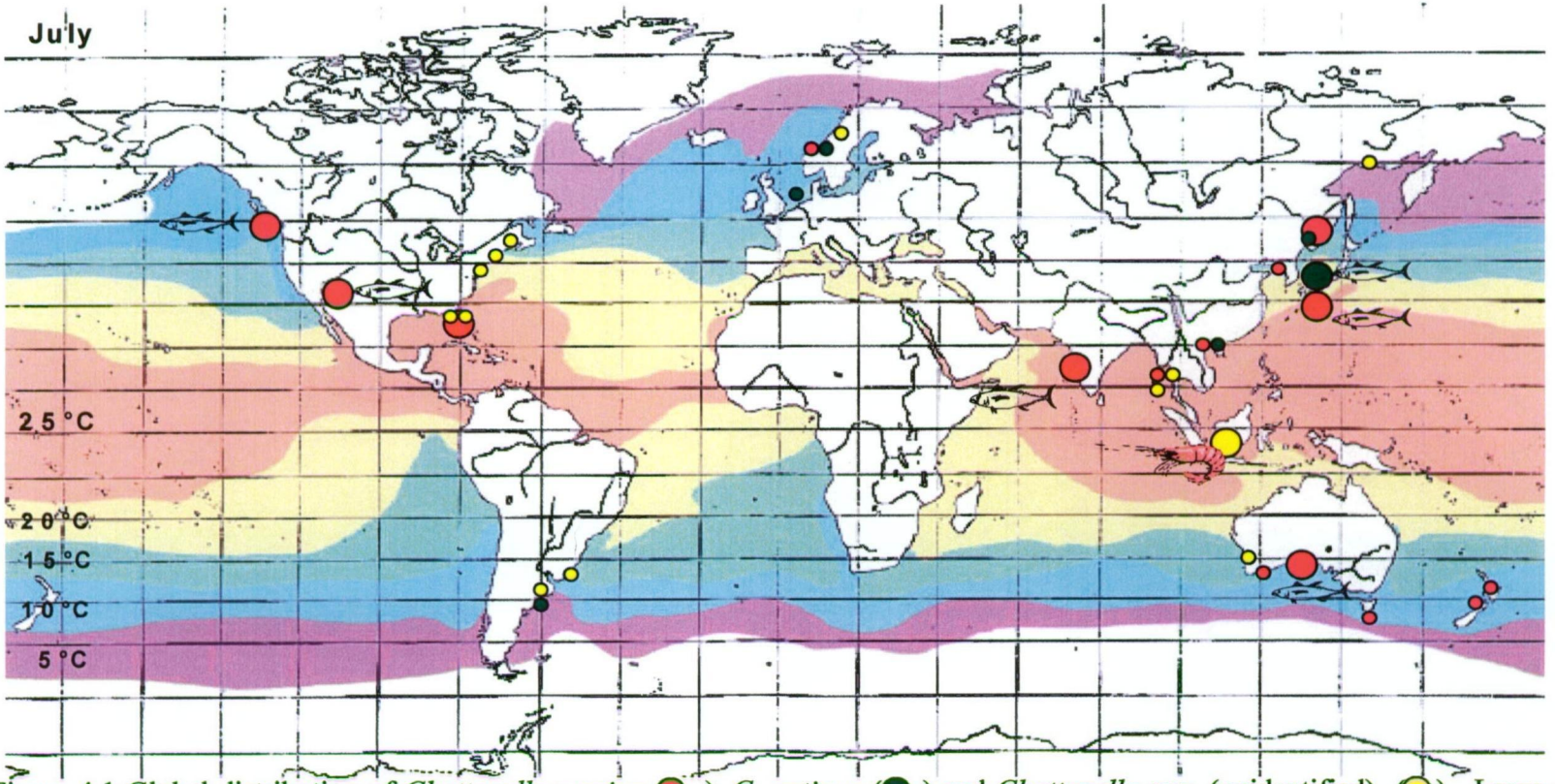
Raphidophyte ecology has been poorly researched hence, this chapter tries to define global patterns in known distribution and ecophysiology of the raphidophytes in order to determine some basic parameters for bloom occurrence.

4.2 *Chattonella marina*

4.2.1 Global Distribution

Chattonella marina is reported from temperate to tropical waters of the Northern and Southern Hemispheres (Fig 4.1). The most frequent occurrence of *C. marina* is from latitudes 25-35° N, in the Asia-Pacific region including China (Tseng *et al.* 1993, Yuzao *et al.* 1993), Korea (Park *et al.*1991), Japan (Okaichi 1997) and USA (Tiffany and Hurlbert 1998; Tiffany *et al.* 2001; Tomas 1998). Sightings have also been made as far North as latitude 52° in the Netherlands (Vrieling *et al.* 1995) and West Canada. In the Southern Hemisphere, *C. marina* has been known to bloom from latitude 25-35°S in Brazil (Odebrecht and Abreu 1995) and Australia (Hallegraeff *et al.* 1998) but also reported at 42°S in Hobart, Australia (Jameson and Hallegraeff pers. comm.) and Wellington Harbour, New Zealand (Chang, 2000).

There are also reports of *Chattonella* sp. (possibly *C. marina*) in tropical climates (0-12°N) in Singapore (M. Holmes pers. comm.), Thailand (Anon. 1997), the Philippines (R. Azanza pers. comm.) and Malaysia (Khoo 1985; Anon. 1997), but no subsequent verification of identification. The first report of *C. marina* (Subrahmanyan 1954, initially described as *Hornellia marina*) was on the tropical Malabar coast of India (25°N). Such distribution points to the existence of cold and warm water ecotypes as discussed later and in Chapter 5.



3.

Figure 4.1 Global distribution of *Chattonella marina* ( ), *C. antiqua* ( ) and *Chattonella* spp. (unidentified) ( ). Large circle denotes bloom, small circle denotes sighting, mortalities denoted by fish ( ) or shrimp ( )

4.2.2 Ecophysiology

Chattonella marina generally requires water temperatures above 23°C in Japan (Imai *et al.* 1998) but bloom conditions of *C. marina* have been reported to range from 17-22°C in Brazilian, Australian and northwest Asian regions, and at 14°C in Western Canada (Floyd Cole pers comm.). This species has also been observed in temperature ranges of 18.8 - 28.0 °C in the Seto Inland Sea (Imai *et al.* 1998). Australian strains are known to tolerate temperatures from 10 - 30 °C in laboratory conditions (Marshall and Hallegraeff 1999; Chapter 5) and blooms have been reported in much warmer waters (Tomas, 1998; 28°C Florida, USA). Reports of *C. cf. marina* in Manila Bay (Philippines) have been associated with cold water currents (around 25°C) possibly from Japan (Kuroshio Current) during El Niño events (R. Azanza pers. comm.) and *C. marina* is not considered to be part of the normal flora of the Philippines. Salinity reported for blooms is usually close to undiluted seawater at 31-32 psu. Blooms have been reported to occur mainly in the summer and autumn months for both the Northern and Southern hemispheres (Table 4.1).

Differences between Australian and Japanese strains in light requirements have been reported (Marshall & Hallegraeff 1999, Chapter 5). A South Australian strain had a higher irradiance tolerance (>1200 µmols photons $m^{-2}s^{-1}$) and light saturation level (400 µmols photons $m^{-2}s^{-1}$) than a Japanese strain (< 400 µmols photons $m^{-2}s^{-1}$; 150 µmols photons $m^{-2}s^{-1}$respectively) in laboratory experiments (Table 4.2). The high light adaptation of the Australian strain has been related to increased mean solar radiation, which South Australia receives during the summer months (Marshall and Hallegraeff 1999; Chapter 5). Further analysis of the Japanese and Australian strains have revealed different physiological adaptations to high irradiance and UV radiation. The Australian strain produced high levels of sunscreens such as mycosporine-like amino acids (MAAs) while the Japanese strain utilised a violaxanthin – zeaxanthin cycle for high irradiance protection (Marshall & Newman, 2002; Chapter 6).

Table 4.1. Occurrence and conditions for blooms of raphidophytes as reported in the literature and from personal communications.

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Chattonella antiqua	Ismael & Halim, 2000	Alexandria	Egypt	1999	No			May-July	33.2	
	Chihara, 1983	Seto Inland Sea	Japan							
	Nakamura *et al.*, 1988	Seto Inland Sea	Japan			$2.5x10^5$			30.0-32.8	24.2-28.1
	Okaichi, 1989	Seto Inland Sea	Japan	1982	Yes	$7.7x10^6$	Summer	August		
	L. Nguyen pers comm.	shrimp ponds	Vietnam	1987	Yes/ shrimp					29
	Odebrecht & Abreu, 1995	Patos lagoon	S Brazil	1993		$35 x10^3$	Autumn	May	Estuarine	19.0-19.5
	Park, 1991	Chinhae Bay	Korea							
	Tomas 1998	Florida Bay	USA		Poss	s	mid-summer		low	
	Vrieling *et al*, 1995	Dutch Coast	Nether-lands							
Chattonella globosa	Chang,1999	Wellington Harbour	New Zealand	1999	No	10^3	Autumn	March-April		
	Hallegraeff & Marshall unpub.	South Australia	Australia	2000	No					
	Marshall present work	Canning River, WA	Australia	2001	No		Autumn	May	32	20
	Hallegraeff & Hara, 1995		Canada							
	Hallegraeff & Hara, 1995		SE Asia							

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Chattonella globosa	Hara *et al.*, 1994	Tokyo Bay	Japan	1991	No	$4.05x10^5$	summer	Oct-Jan	20-31	20->8
	Songhui and Hodgkiss	Dapeng Bay	China	2001	No		Spring	April		
Chattonella ovata	Hara *et al.*, 1994	Western Japan	Japan	1990						
	Songhui and Hodgkiss	Dapeng Bay	China	2001	No		Spring	April		
Chattonella marina	Ono *et al.* 1996	Seto Inland Sea	Japan	77-87	Yes					
	Chihara, 1983	Seto Inland Sea	Japan							
	Yoshimoto & Ono, 1986	Seto Inland Sea	Japan							18.8-28
	Hallegraeff *et al.*, 1998	South Australia	Australia	1996	Yes		Autumn	April	35.3-36.9	17.0-17.7
	Hallegraeff & Jameson unpub.	Derwent Estuary	Australia		No		late summer	March		
	Marshall present work	Port Lincoln	Australia	2001	No	250	Autumn	May	34	17
	M. Holmes pers. comm.		Singapore		No					
	Khoo, 1985	Johor Straits	Malaysia	1983	Yes/ shrimp			Mar-Nov		
	Park, 1991	Chinhae Bay	Korea			$4.2x10^4$				
	Chang 2000	Wellington Harbour	New Zealand	2000	No		Summer	Jan-Feb		
	Rhodes & Thomas, 1997	Rangaunu Harbour	New Zealand	1997		10^3	Autumn	April		
	Subrahmanyan, 1954	Malabar Coast	India	1954	Yes					
	Tiffany *et al.* 2001	Salton Sea	USA	1997	Yes	$6.0x10^5$		Nov	50	30

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Chattonella marina	Tomas 1998	Florida Bay	USA	90-95		$>10^6$	late summer	Jul-Aug	<32ppt	>28
	Tomas 1998	Tampa Bay	USA	90-95		$>10^6$	late summer	Apr-May	<32ppt	>28
	Haigh pers comm..	Esperanza Inlet	Canada	2002	Yes	5.0×10^4	Autumn	Sept	35	13.8
	Tseng *et al.*, 1993	Dapong Bay	China	1991				March		
	Yuzao *et al.*, 1993	South China Sea	China	1991				March	32->31	32-31
	Vrieling *et al*, 1995	Dutch Coast/ N Sea	Netherlands	91-93	No	2.5×10^5		Mar-Jun		
Chattonella sp.	Anon, 1997	Gulf of Thailand	Thailand							
	Anon, 1997	Straits of Johor-east	Malaysia		No					
	R. Azanza pers comm.	Manila Bay	Phillipines	97-98	No					25
	Elder & Henroth, 1999		Norway	1998						
	Lu and Göbel	German Bight	Denmark	2000	Yes	8.7×10^6	Spring	May		
	W. Hosja pers. comm.	Wilsons Inlet, WA.	Australia	1997	No		Summer	Dec	17	
	Imai et al. 1998	Hiroshima Bay	Japan							
	Lirdwitayaprasit *et al.*, 1996	Chantiburi	Thailand	1995	Yes/ shrimp					
	Odebrecht & Abreu, 1995	Rio de Janero	S Brazil	1978	No	17×10^3		Jun-Jul		
	Orlova *et al.*, 1998	Peter the Great bay	Russia	1987	Yes			Sept		
	Tomas, 2000	Corpus Christ, Texas	USA	2000				June-Sept		

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
	Tomas, 2000	Bayboro Harbour, Florida	USA	2000				June-Sept		
Chattonella sp.	Tomas, 2000	St Johns River Estuary, Florida	USA	2000				June-Sept		
	Tomas, 2000	New/Neuse River, North Carolina	USA	2000				June-Sept		
	Tomas, 2000	Ayre Creek, Maryland	USA	2000				June-Sept		
	Tomas, 2000	Bald Eagle Creek, Delaware	USA	2000				June-Sept		
Chattonella subsalsa	Mignot, 1976	France to Algeria	Mediterranean							
	Tomas 1997	Florida Bay	USA			$>10^6$		Apr-May	<32	>28
	Tomas 1997	Tampa Bay	USA	90-95		$>10^6$	early summer	Jul-Aug	<32	>28
	Domingos & Menezes, 1998	Barra lagoon	Brazil	90-93				Jan	7.1	
Chattonella sp. (? verrucculosa)	Anon, 2001		Norway	2001	Yes			March		
	Dahl pers.comm..	Southern coast	Norway	2001						
Chattonella verruculosa	Horstmann et al., 1998	Flekka fjord	Norway	1998	Yes	$2.30x10^7$		Early May		
	Kass, 1998	Flekka fjord	Norway	1998	Yes		late spring	May		
	Imai et al., 1996a	Hiroshima Bay	Japan	1993	Yes		summer	May		
	Yamamoto & Takana, 1990	Seto Inland Sea	Japan	1989	Yes		summer		32.6-32.8	12.3-12.7
	Phycotoxins newslist	Flekka fjord	Denmark	1998	Yes	$1.00x10^7$		Late May		

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Fibrocapsa japonica	Billard, 1992	French coast	France							
	Chihara, 1983	Seto Inland Sea	Japan							
	Hallegraeff & Hara, 1998		Japan							
	Cosgrove pers comm.	Swan River, WA	Australia	2001	No	$1.59x10^5$	Autumn	May	22-32	19
	Marshall present work	Derwet Est, Tas	Australia	2001	No		Autumn	May		14
	M. Elbrachter pers. comm.	German bight								
	Vrieling *et al*, 1995	Dutch Coast	Netherlands			1x10.4 l				
	Kass, 1998	Flekka fjorn	Norway	1998			late spring	May		
	Lee *et al*. 2000	Kamak Bay	Korea	1998	No			June		
	Loeblich & Fine,1977	California	USA	1970				July		
	P. Hargraves pers. comm.	Narragansett Bay	USA		No	10^4	Summer		18-28	25
	Tomas 1998	Florida Bay	USA			10^5		May-Aug	<32ppt	>28
	Tomas 1998	Tampa Bay	USA	90-95	10.5	May-Aug	<32ppt	>28°C		
	Odebrecht & Abreu, 1995	Patos Lagoon	Brazil	1995	No	$4.8x10^4$	Autumn	May	Estuarine	19.0-19.5
	Rhodes *et al*., 1993	North coast	New Zealand	1992	No	$1.1x10^5$	Spring	Oct	35	14.5-19
	Reid *et al*., 1990	Belgium coast	Belgium							
Haramonas dimorpha	Horiguchi, 1996	Cape Tribulation	Australia							

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Haramonas sp.	Hallegraeff pers comm.	Parramatta River	Australia	1998	Yes		Early Summer	Dec		
Heterosigma akashiwo	Gowen 1987		Scotland	1979	Yes					
	Anon, 2001	Kagoshima Bay	Japan	2001	Yes			March		
	Chihara, 1983	N & S Islands	Japan							
	Honjo	throughout	Japan							
	Anon, 1997	Gulf of Thailand	Thailand		Yes					
	Anon, 1997	Straits of Johor	Malaysia		Yes					
	Chang et al. 1990, MacKenzie, 1991	Big Glory Bay	New Zealand	1989	Yes	2x10.6 l	Summer	Jan	33.8-34.3	15-15.5
	Chang 1999	Wellington Harbour	New Zealand	1999	No	8.20E+07	Summer	Jan		
	Rhodes *et al.* 1993	Rangaunu Harbour	New Zealand	1997			Summer	Feb		
	Rhodes *et al.*, 1993	North coast	New Zealand	1992	Yes		Spring/ Summer	Aug-Dec	35	14.5-19
	P. Christy pers comm.	Port River, Port Lincoln, SA	Australia	yearly	?		Summer			
	Hallegraeff & Marshall unpubl.	Gold Coast, Qld	Australia	2000	Yes		Autumn	May		
	Clement & Lembeye 1993	35° to45°S	Chile							
	Tomas 1980	Narragansett Bay	USA	Yearly 1966 ->	No		early summer			
	Tomas 1998	Florida Bay	USA	89,91		>10 .6		Apr-May	<32ppt	>28
	Tomas 1998	Tampa Bay	USA	87-88		>10 .6	late summer	Jul-Aug	<32ppt	>28
	Tomas 2000	Padri Island,Texas	USA	99-2000	Yes					

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Heterosigma akashiwo	R. Horner pers comm.	Washington	USA	1989	Yes			Sept		
	Horner et al.,1997	Pacific Coast	USA & BC		Yes					
	Li & Smayda, 2000	Narragansett Bay, RI	USA	59-96				May-Jul, Oct-Nov		Key factor
	Smayda 1997	North of 40°	USA							
	Loeblich & Loeblich	New York	USA	1963						
	Rensel, 1995	Puget Sounds	USA	1990	Yes			Jul		
	Gaines & Taylor, 1986	British Columbia	Canada	Yearly	Yes			May-July		
	Konovalova 1995	55° to 30° N	Pacific							
	Orlova et al., 1998	Peter the Great Bay	Russia	95/96		5.00E+06		Jun-Jul		
	News	SW Coast	Norway	98	Yes		late spring	May		
	Phycotoxins Newslist, 15/5/98	Southern Coast	Norway	1998	Yes			Early May		
	Rademaker et al., 1995	Dutch Wadden Sea	Germany	1994	No	1.00E+06				
	Smayda 1997	40° N to 55°N	Europe							
	Smayda 1997	Portugal to Oslofjord	Europe							
	J. Silke pers. comm.	Coast of Galway	Ireland	1980's	Yes			May-June		15->
	Smayda 1997		Nambia							
	Lirdwitayaprasit et al., 1996	Chantaburi	Thailand	1995	Yes/ shrimps					

Species	Reference	Location	Country	Year	Toxic	Density (cells L^{-1})	Season	Month	Salinity ppt	Temp °C
Heterosigma akashiwo	Tomas 1980	Need confirmation	Bermuda							
	Park 1991		South Korea							
	Park, 1991	Bukshin Bay	Korea	83-86		10x10.4 ml		May-Jul		
	Procenca 2001	Paranagua Bay	Brazil	2001	Yes		Spring	March		
	Qi et al. 1993	coastal waters	China							
	Tseng et al. 1993	Dalian Bay	China	1985						
	Yuzao et al., 1993	Dalian Bay	China	1985-88						

Chattonella marina is known to produce cysts as an overwintering survival strategy in Japanese waters. *Chattonella* cysts start to germinate from shallow coastal areas where the bottom water temperature is above 20°C in early summer, with germination extending to deeper waters as the bottom water temperature rises during the season (Imai *et al.* 1998). Hence *Chattonella* appears to have a germination period over 3-4 months which enables protracted bloom formation. Cysts of *Chattonella* spp. have also been noted in Korea during January –April (winter) and July-August (Summer) (Lee *et al.* 2000). The South Australian strain has the ability to produce cysts under laboratory conditions (Marshall unpublished data) but only sparse *Chattonella* spp. cysts have been located in South Australian waters (Bolch and Hallegraeff pers. comm.).

Most nutritional studies have been reported on Japanese strains of *C. marina*, which may be a different ecophenotype and have varying nutritional requirements compared to strains isolated from other global locations. Studies of chemical requirements for growth suggest that the Japanese strains of *C. marina* may have a higher nitrogen and phosphorus requirement than *C. antiqua* (Ono 1988). *Chattonella marina* blooms are mainly noted around areas of intensive aquaculture, suggesting that organic pollution may stimulate blooms.

Yuzao *et al.* (1993) found that blooms of *C. marina* in China were preceded by an increase in ammonium and phosphate concentrations in deep water, possibly due to water column overturn. Cells exhibited a ten-fold increase in concentration, far higher than that predicted from measurements of ambient nutrient levels before or during the outbreak. He explained this from the organism's ability to perform active vertical migration, actively aggregating at a position in the water column and/or secondarily accumulated by physical factors such as fronts. During the build-up of the bloom, nitrate, ammonium and phosphate concentrations in the surface waters increased rapidly and salinity decreased from 32 to 31 psu. *C. marina* blooms appeared in the surface water not only because of suitable physical conditions for growth, but also were concentrated

Table 4.2 Comparison of growth parameters between Japanese and Australian strains of *Chattonella marina* under laboratory conditions.

	Australia Port Lincoln, SA	Japan Seto Inland Sea
Strain Designation	UTCMPL01*	NIES-118**
Optimal growth rate (divisions/day)	1.08[1]	0.75[1]
Optimal light intensity (μmols photons $m^{-2}s^{-1}$)	400[1]	150[1]
Photoinhibition (μmols photons $m^{-2}s^{-1}$)	1200[1]	400[1]
Average summer sunshine (hours per day)	7.9[2]	6.8[3]
Average summer mean solar global radiation ($MJ.m^{-2}$)	26.8[2]	18.3[3]
Light penetration (Secchi depth m)	6-10[4]	4[5]

*Harmful Algae Culture Collection, University of Tasmania, Australia; **Microbial Culture Collection of the National Institute of Environmental Studies, Tsukuba, Japan; [1]Marshall & Hallegraeff 1999, [2] Australian Bureau of Meterology; [3]Japanese Meteorological Agency; [4]Clarke 1996; [5]Hashimoto et al. 1997.

onshore by moderate onshore winds and tidal currents in Dampong Bay, China (Yuzao *et al.*1993). Cell numbers fluctuated with tidal currents. Wind and tidal concentration of *C. marina* blooms have also been noted in Japan (Ono *et al.* 2000) and are thought to have been a contributing factor in the 1996 bloom in Port Lincoln, South Australia.

Chattonella blooms usually occur when diatoms are scarce (< 10^2 cells per ml in the surface waters, Yoshimatsu and Ono 1986). Low silicon levels and subsequent limited diatom growth may be crucial in the development of *Chattonella* blooms as demonstrated in Fig 4.2.

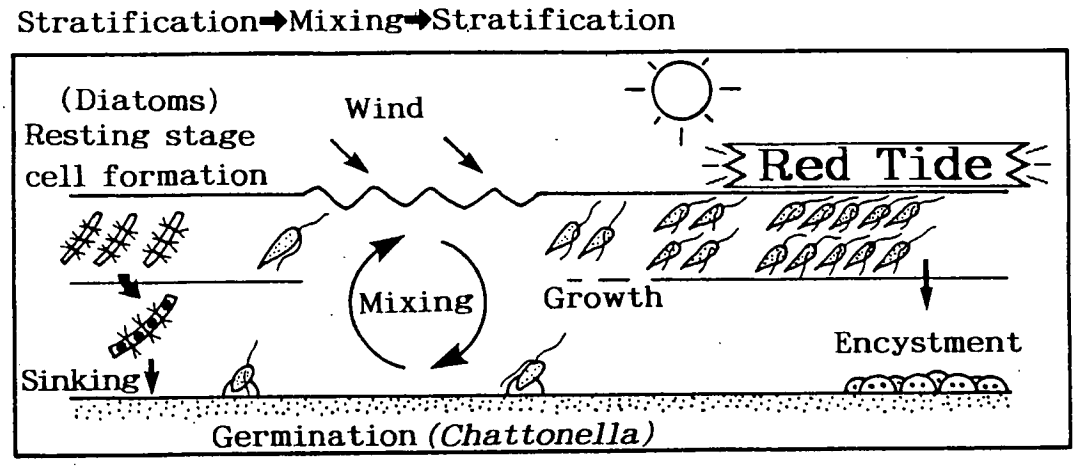


Figure 4.3. A schematic representation of the scenario leading to *Chattonella* blooms in the Seto Inland Sea in summer (adopted from Imai 1998).

Growth yield of *C. marina* in laboratory studies is promoted by iron-EDTA and phosphorus, especially in combination. Nitrogen is only effective after enrichment with iron-EDTA and phosphorus, suggesting that chelated iron is a crucial factor triggering *C. marina* blooms (Yamochi 1984b). Iron may also play a crucial role in the toxicity of *C. marina*, influencing the rate of reactive oxygen species (ROS) production. Known physiological properties of *C. marina* are listed in Table 4.3

Subrahmanyan (1954) first reported *Chattonella (Hornellia) marina* as occurring "after the stormy monsoon season when water agitation stirred up the muddy sea bottom and fine suspension of mud particles imparted an amber or ocherous colour". This colouration of the water has subsequently been reported for other *Chattonella* blooms including Boston Bay in April 1996 (Clarke *et al.* 1996), after a "surge event" which suspended fine bottom sediment in the water column. The suspension of sediments may result in increased phosphates and iron acting as a bloom stimulant.

Table 4.3. Physiological properties of *Chattonella marina*, summarized from the literature.

CELL CHARACTERISTICS			
Cell size	30 - 70 µm long		
	20 - 30 µm wide		
Chlorophyll	3.1 – 14.88 pg.cell^{-1} [1]		
CELL QUOTAS (q_0)			
C	7.38 – 10.31 ng.cell^{2}	C:N	15:2 (exponential)[3]
N	0.99 – 1.59 ng.cell^{2}	C:N	13:2 (stationary)[3]
P	6.64 – 119.38 ng.cell^{2}	N:P	25:3 (exponential)[3]
		N:P	239:1 (stationary)[3]
PHOTOSYNTHESIS - GROWTH			
I_c Japan	25 µmol m^{-2}s^{-1} [1,4]	I_{sat} Japan	110 µmol m^{-2}s^{-1} [1,4]
I_c South Australia	50 µmol m^{-2}s^{-1} [5]	I_{sat} South Australia	400 µmol m^{-2}s^{-1} [5]
TEMPERATURE & SALINITY TOLERANCES			
°C optimum	25[4,5]		
range	10 - 30[4,5]		
psu optimum	20 (Japan)[1]		
	30 (South Australia)[5]		
range	15-50[5]		

Source; [1]Nakamura and Watanabe 1993a; [2]Lirdwitayaprasit et al. 1996; [3]extrapolation from Lirdwitayaprasit et al. 1996; [4]Yamaguchi et al. 1991; [5]Marshall and Hallegraeff 1999.

Global trends in *C. marina* bloom formation are difficult to establish due to the scarcity of physio-chemical data such as water temperature and salinity, and nutrient status. Japan has provided the majority of research into the physiology of this species, but recent findings by the author (Chapter 5 and 6) suggests that triggers for bloom formation may vary between continents due to ecophenotypic differences. However, consistent factors for *C. marina* blooms are;

- a surge or storm event to suspend sediment and organics
- some type of organic pollution as a stimulus

4.3 *Chattonella antiqua*

4.3.1 Global Distribution

Chattonella antiqua has a similar but more restricted global distribution than *C. marina* (Fig 4.1) This species predominates in the Asia-Pacific region of Japan (Okaichi *et al.* 1989) and Korea (Park 1991) between latitudes 30° and 40°N but also has been reported by Vrieling *et al.* (1995) on the Dutch coast (Lat 55°N), Florida Bay, USA (Lat 25°N; Tomas 1998) and in the Southern Hemisphere in Patos Lagoon, Brazil (Lat 25°S; Odebrecht and Abreu 1995). Only low numbers of *C. antiqua* have been reported outside Asian-Pacific waters and these usually co-occurred in a bloom of *C. marina* (Tampa Bay, Tomas 1998; Patos Lagoon; Odebrecht and Abreu 1995, Dutch Wadden Sea; Vrieling *et al.* 1995). It may be possible, particularly in the colder waters, that the cells identified as *C. antiqua* could be larger planozygote cells of the more widely distributed *C. marina.* This would leave *C. antiqua* with a restricted distribution around Japan and Korea in sheltered embayments as discussed later.

4.3.2 Ecophysiology

Physical conditions for Japanese blooms of *C. antiqua* are similar to those for *C. marina* occurring at 24-28°C temperature and 30-32 psu salinity (Imai 1998, Nakamura and Watanabe 1983a). These two *Chattonella* species often coincide

in the Seto Inland Sea, Japan. However, reports of *C. antiqua* in the comparatively cold waters of Patos Lagoon, Brazil (19°C) and the Dutch Wadden Sea have not yet been substantiated. Blooms have been reported in shrimp (*Penaeus monodon*) ponds during 1997 in Thailand in waters of 29°C (Nguyen Ngoc Lam, pers. comm.). Blooms usually occur during summer and autumn in the Northern Hemisphere.

Watanabe *et al.* (1995) observed diel vertical migration and nocturnal uptake of PO_4^{3-} and nitrogen by *C. antiqua* in the nutrient rich lower layer of a stratified tank, analogous to the nutrient conditions in a natural *Chattonella* red tide. Nutrients could be taken up in the dark at a rate almost equal (83-93%) to that in the light (Nakamura and Watanabe 1983c). This species uses nitrate, ammonium, and urea, but not amino acids (glycine, alanine and glutamate) as nitrogen sources (Nakamura and Watanabe 1983b). *Chattonella antiqua* shows a chemotactic response to levels of inorganic phosphate between 10^{-3} to 10^{-6} M (Ikegami 1995, Nakamura and Watanabe 1983b) as well as a dependence on Fe (Nakamura 1990, Okaichi 1993) and a requirement for vitamin B_{12} (half saturation constant of 0.1-0.35 ng l^{-1}; Nishijima 1986).

Accumulation of *Chattonella antiqua* cells in the surface layers due to upward migration during the daytime was noted by Watanabe (1995). This vertical migration indicates that *C. marina* and *C. antiqua* have co-specific bloom requirements. It has been proposed that influxes of large amounts of nutrient-laden fresh water act as a precursor to *C. antiqua* blooms (Fukase 1980, Watanabe 1995). Mathematical modeling of environmental factors such as the presence of a shallow nutricline at 5-7 meters, reduced water exchange to aid stratification, nutrient poor surface waters and an initial concentration of cysts may be driving forces for a *C. antiqua* bloom (Amano *et al.* 1998).

4.4 Other *Chattonella* species

4.4.1 Global distribution of *Chattonella globosa*

Chattonella globosa has been reported in Japan (originally as *Chattonella* sp. globular type; Hosaka *et al.* 1991) as well as Canada, south-east Asia (Hallegraeff and Hara 1995) and New Zealand (Chang 1999). It has recently been observed using light microscopy by the author in South and Western Australian waters (Fig 4.4). This species is thought to have a temperature tolerance lower than *C. antiqua* and *C. marina* , of 8 to 20 °C and a salinity range of around 22-32 psu. (Hosaka *et al.* 1991), but little work has been done to define its physical requirements for growth.

4.4.2 Global distribution of *Chattonella ovata*

Chattonella ovata has only been unambiguously reported in Japanese waters. Despite physiological and molecular research on isolated strains, there are is only one reported sighting of this species since the original by Yoshimatsu and Ono (1986) in Southern China waters (Songhui and Hodgkiss 2001). (Fig 4.4).

4.4.3 Global distribution of *Chattonella subsalsa*

Chattonella subsalsa has been reported to bloom in the western Mediterranean from France to Algeria (Biecheler 1937, Holland 1956, Mignot 1976, Fig 4.4). There is no record of its occurrence in the Southern Hemisphere. Blooms reported in the USA occur in full or high salinity waters (32-50 psu) and temperatures of 24-34 °C in early summer (Tomas 1998). The bloom in the Port of Algiers was reported to occur at 24°C and 37 psu salinity (Biecheler 1937).

4.4.4 Global distribution of *Chattonella verruculosa*

Until recent times, there have been few reports of *Chattonella verruculosa*. An ichthyotoxic bloom was first reported in the Seto Inland Sea, Japan in the late 1989 (Yamamoto and Tanaka 1990). The species was also isolated from

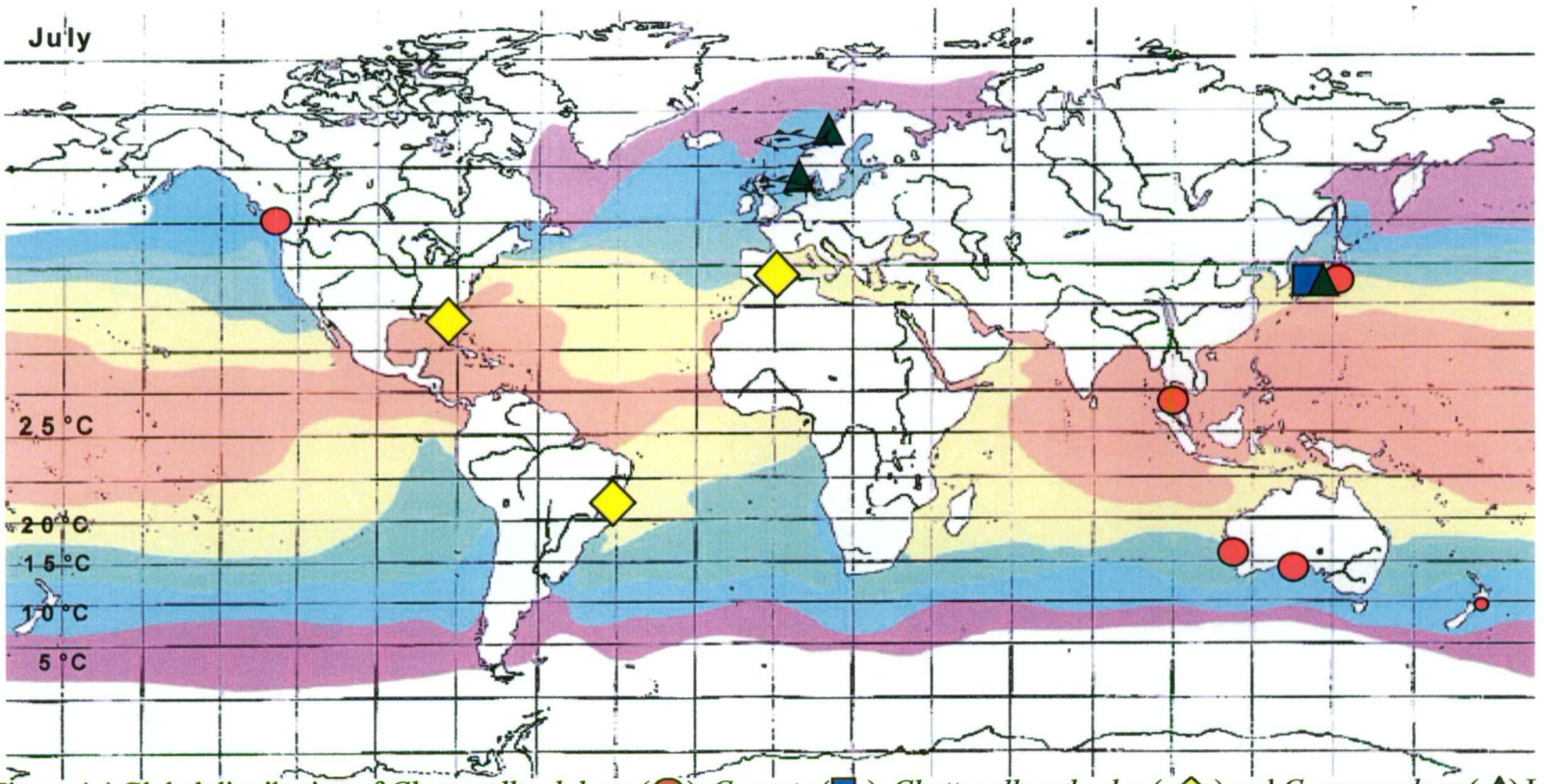


Figure 4.4 Global distribution of *Chattonella globosa* (●), *C. ovata* (■), *Chattonella subsalsa* (◆) and *C. verruculosa* (▲).Large symbol denotes bloom, small circle denotes sighting

Hiroshima Bay, Japan, by Imai (1996a) in 1993. In 1998 in Norway a large bloom was tentatively identified as *C. verruculosa* (Kaas 1998), and associated with mortalities on salmon farms and wild fish populations (Back-Hansen *et al.* 2000). This species is now claimed to be a permanent part of the marine flora in the area (Solholm 2001). No sightings of *C. verruculosa* have been reported in the Southern Hemisphere (Fig 4.4). Blooms have been reported in colder waters (18.8°-21.5°C temperature), than for other *Chattonella* species. Growth of this species is potentially limited by the availability of selenium (Imai *et al.* 1996a). The bloom on the Norwegian/Danish coasts was associated with nutrient rich water transported in the Jutland current (Kaas pers comm.), and it is suggested that anthropogenic nutrients most likely are stimulating the blooms in the North Sea (Aure *et al.* 2001).

4.5 *Heterosigma akashiwo*

4.5.1 Global Distribution

This species has a widespread distribution throughout the Northern and Southern Hemispheres (Fig 4.5). Major blooms have occurred in the North Pacific ocean on the West coast of America and Canada (Gaines and Taylor 1986, Konovalova 1995, Smayda 1998, Tomas 1998,), in the Asia Pacific region (Honjo 1993, Orlova 1997, Park 1991, Qi 1993, Tseng 1993, Yuzao 1993), the North Atlantic USA and Western European coasts (R. Horner pers. comm., Tomas 1998). The reported distribution in the North ranges from latitudes 20-55°N, with isolated unconfirmed sightings in the sub-tropical/ tropical waters of Bermuda (Tomas 1982), Taiwan, Singapore (Smayda 1998), Thailand (Lirdwitayaprasit 1996) and also Ireland (J. Silke pers. comm.). Distribution in the Southern Hemisphere is as widespread with blooms occurring around Peru and Chile (Clement 1993), Southern Africa (Smayda 1998) and in the Australasian region in latitudes 15-

40°S (Chang 1990, Hallegraeff 1991, Mackenzie 1991, Rhodes 1993, Smayda 1997).

3.5.2 Ecophysiology

H. akashiwo has been known to bloom in waters of 20 to 25°C and in culture can grow up to 30°C (Yamochi 1984a), hence subtropical and tropical habitats would appear suitable for blooms of this organism. The required minimum temperature for "over-wintering" quiescence (from sexual cysts) is < 10°C (Yamochi 1986). Such conditions occur in the Northern Hemisphere, in the Asia-Pacific region (Japan, Korea, China), Eastern and Western Atlantic coasts and the West Coast of Canada. There have also been blooms of *Heterosigma* in British Columbia waters every year since the 1960s. Fish kills have been reported most years since 1986 in British Columbia and in some years (1989, 1990) in Washington (Horner *et al.* 1997).

The Southern Hemisphere has a similar pattern of blooms occurring in areas where winter temperatures are between 10 and 15 °C. Most blooms are reported to occur during the austral summer months. Sub-tropical water observations have been reported in Brisbane, Australia (Hallegraeff pers. comm.), Florida (Tomas 1998), Singapore (Taylor 1993 unconfirmed) and Thailand in a shrimp pond co-occurring with *C. marina* (Lirdwitayaprasit *et al.* 1996). Light microscopic observations of *H. akashiwo* may be insufficient to confirm species identity. Gametes of other raphidophyte species, such as those of *C. marina*, are very similar in size and morphology to *H. akashiwo*, and therefore may be mis-identified. Major blooms of *H. akashiwo* indicate it is predominantly a cold temperate species. Smayda (1998) noted a remarkably persistent relationship between temperature and *H. akashiwo* blooms throughout its distribution range. Blooms cluster within temperature ranges of 15-20°C, indicating that temperature is a major factor in the growth and seasonal bloom cycle of this species (Fig 4.6).

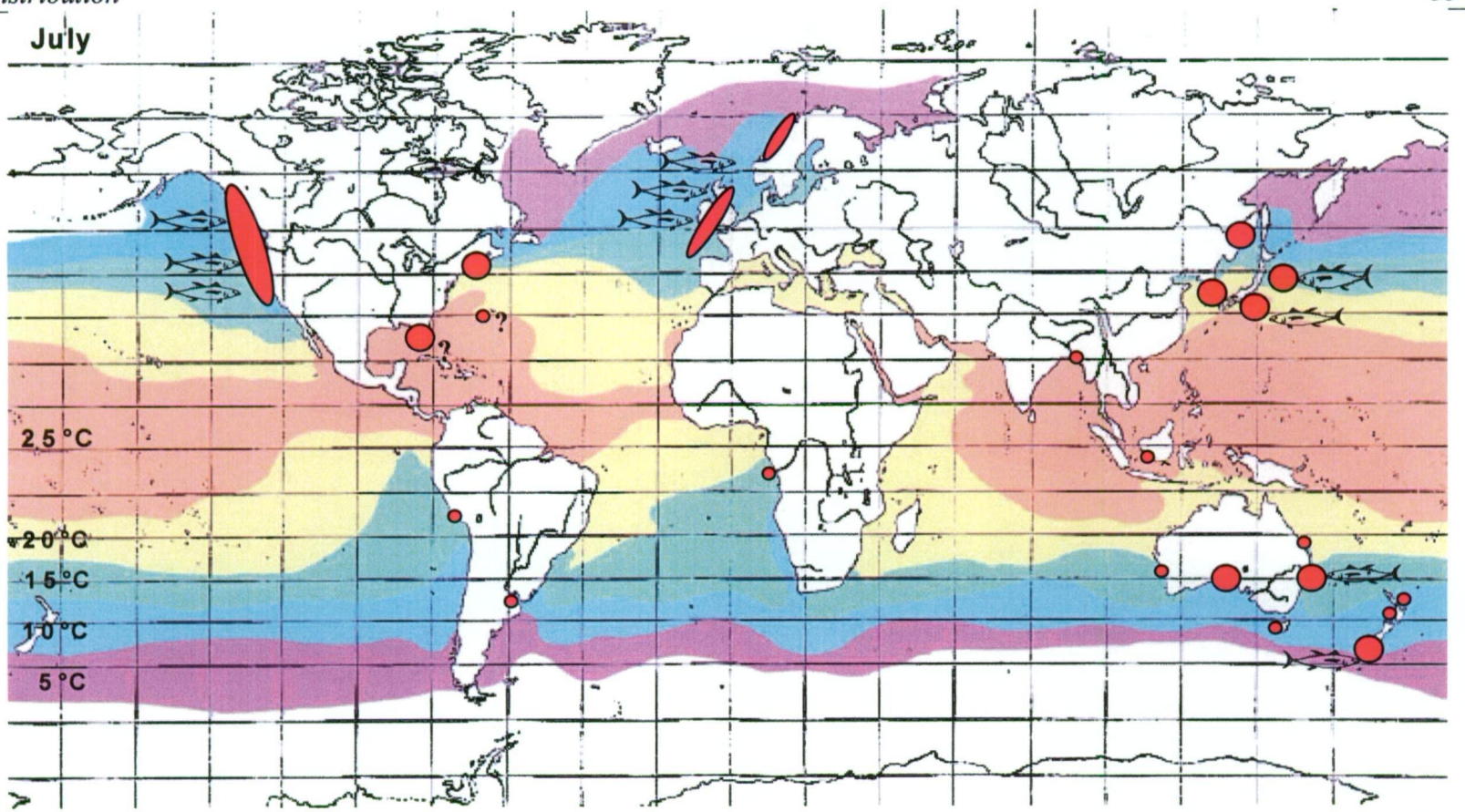


Figure 4.6 Global distribution of *Heterosigma akashiwo* Large circle denotes bloom, small circle denotes sighting, mortalities denoted by fish ( )

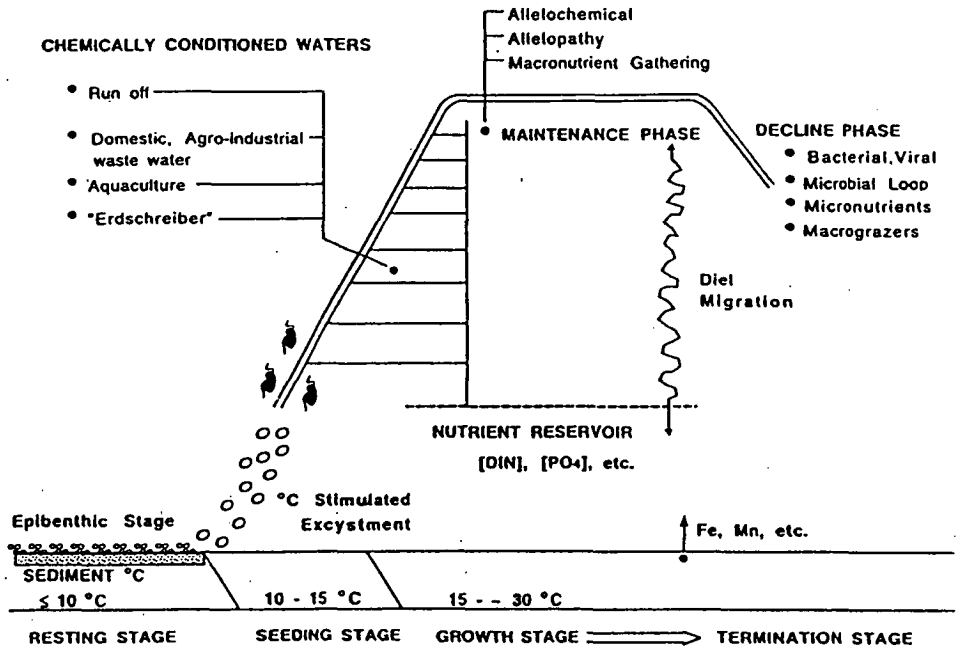


Figure 4.6; General model of the key processes influencing blooms of *Heterosigma akashiwo* (adopted from Smayda 1998).

In the Seto Inland Sea, the frequency of occurrence of *H. akashiwo* is closely related to eutrophication (Honjo 1993). Blooms require metals, such as iron and manganese, in addition to nitrogen, phosphorus and vitamin B12 (Watanabe *et al.* 1982). *H. akashiwo* has a high growth potential of up to 5 divisions per day. Historical data on nutrient loading of the Seto Inland Sea show a positive correlation between eutrophication and the number of *Heterosigma* blooms (Honjo 1993). *Heterosigma* has relatively high nutrient requirement (Table 4.4).

Hershberger (1995) has shown from laboratory experiments that *Heterosigma* responds to salinity gradients, migrating to the surface of test tubes upon addition of a thin layer of freshwater. Moreover, cells remained above the halocline, possibly by changing their density. Thus a bloom may require heavy rainfall or runoff followed by mild weather with little wind which allows the water column to stratisfy long enough for the cells to accumulate at the surface (Hershberger

1995; Hershberger *et al.* 1997). This phenomenon has been demonstrated by blooms occurring in water bodies in calm weather after heavy rain (Strait of Georgia; Taylor and Haigh 1993, Puget Sound; (Rensel 1995), Stewart Island, (Mackenzie 1991). Nagasaki *et al.* (1996) related the occurrence of a *Heterosigma* red tide in Hiroshima Bay (Japan) to stratification after a rainfall event causing low salinity, higher than average water temperature and increased DIN and DIP. Environmental conditions which supply these nutritive substances include river run-off, bottom water having low oxygen content, and wind induced turbulence of bottom sediments.

Smayda (1998) has described 3 distinct regional populations for *Heterosigma akashiwo* with bloom habitats characterised by one of three different primary features: physically, chemically and biologically dominated habitats. Physically dominated system are regions of intermittent upwellings such as the Peruvian system, Spanish Gallician rias (Mediterranean), Chloe Archipelago (Chile) and Namibian coastal waters where episodic blooms occur after post-upwelling relaxations. During El Niño events where upwelling-relaxation sequences are pronounced and associated with wind, rain and runoff events, such as on the coast of New Zealand, blooms of *H. akashiwo* can be significant (Chang, 1990; Rhodes *et al.,* 1993). Chemically dominated habitats are often nutrient enriched through man-made interventions, such as the Oslofjord (Norway), Tokyo Bay, sub regions of the Seto Inland Sea (Japan), and Masan Bay (Korea). Blooms have been recorded in the nutrient-enriched bays of Japan, Portugal and Korea, where a build up of domestic/industrial waste has preceded its bloom occurrence. However, Smayda (1998) expressed the need to distinguish between macronutrient (N, P) induced blooms from agriculture, sewerage etc. and blooms initiated by micronutrients such as Fe and other trace metals. *Heterosigma akashiwo* blooms have often been linked to river run-off through Fe delivery (Honjo 1993, MacKenzie 1991, Taylor 1993). Yamochi (1989) considered this to be crucial in Osaka Bay (Japan). Biologically dominated habitats stimulatory to

Table 4.4 Physiological requirements of *Heterosigma akashiwo*. DNA, chloroplast number and chlorophyll per cell; V_{max} as pmol $cell^{-1}min^{-1}$, except for Fe given as fg $cell^{-1}h^{-1}$; I_c and I_{sat} are compensation and saturation intensities, respectively; PQ=photosynthetic quotient. (adapted from Smayda 1998).

CELL CHARACTERISTICS			
Cell size	491-1763 μm^3 [1]		
Chloroplasts	4-95 [2]		
Chlorophyll	3.3-5.9 $pg.cell^{-1}$ [3]		
CELL QUOTAS (q_0)			
C	119-429 $pg.cell^{-1}$ [1]	C:N	5.1–7.5:1 (exponential)[6]
N	24 $pg.cell^{-1}$ [4]	C:N	7.1-9.1:1 (stationary)[6]
P	1.82 $pg.cell^{-1}$ [4]	N:P	11-25:1 (exponential)[6]
Fe	5.37 $pg.cell^{-1}$ [5]	N:P	19-41:1 (stationary)[6]
PHOTOSYNTHESIS – GROWTH			
I_c	9 $\mu mol\ m^{-2}s^{-1}$ [3]	PQ	1.43[3]
I_{sat}	0.28 ly min^{-1} [5]	μ_{max}	2.0-5.0 d^{-1} [7]
TEMPERATURE & SALINITY TOLERANCES			
°C	optimum	20[8]	
	range	<20 – >30[8]	
psu	optimum	15-25[8]	
	range	1->50[8]	

Source; [1]Thompson *et al.* 1991; [2]Cattolico *et al.* 1976; [3]Tomas *et al.* 1980; [4]Hosaka 1992; [5]Watanabe *et al.* 1989; .[6]Tomas 1979; [7]Honjo and Tabata 1985; [8]Watanabe *et al.* 1982

H. akashiwo blooms include aquaculture sites, particularly salmonoid fish-farming (Smayda 1998). The presence of the species was often revealed only after the initiation of fish-farming which seemingly triggers fish-killing blooms e.g., British Columbia (Taylor 1993), Chile (Clement and Lembeye 1993), Spain, New Zealand (Chang *et al.* 1990, MacKenzie 1991), Scotland (Ayres *et al.* 1982), United States (Taylor 1993), and Japan (Honjo 1993).

4.6 *Fibrocapsa japonica*

4.6.1 Global Distribution

Fibrocapsa japonica is known to bloom between latitudes 25-40°N and at 25° S, with sightings at 55° N, and 35° S (Fig 4.7). Although there is limited literature on the distribution of *F. japonica*, there exist reports of blooms on an annual basis in Japan (Iwasaki 1989, Toriumi and Takano 1973, Yoshimatsu 1987). It was not recorded to bloom elsewhere until the early 1990's in New Zealand (Rhodes *et al.* 1993), the east coast of the USA (Tomas 1998, P. Hargraves pers. comm.), the French, Dutch and Belgian coasts (Billard 1992, Reid *et al.* 1990, Vrieling *et al.* 1995), German Bight (Elbrachter pers. comm.), Brazil (Odebrecht and Abreu 1995) and Australia. Although *Fibrocapsa* has been shown to produce a neurotoxin lethal to fish (Kahn et al. 1996) blooms have not yet been demonstrated to be toxic (Rhodes 1997, Vrieling et al. 1995). Many reported sightings of *F. japonica* are associated with other raphidophyte blooms such as *H. akashiwo* (Tomas 1998), *C. marina* and *C. antiqua* (Vrieling *et al.* 1995).

4.6.2 Ecophysiology

Blooms of *Fibrocapsa japonica* occur during spring and early summer in both Northern and Southern hemispheres in water temperatures from 14 - 28°C and less than full strength seawater (East Greenwich Cove, USA 18-28 psu salinity;

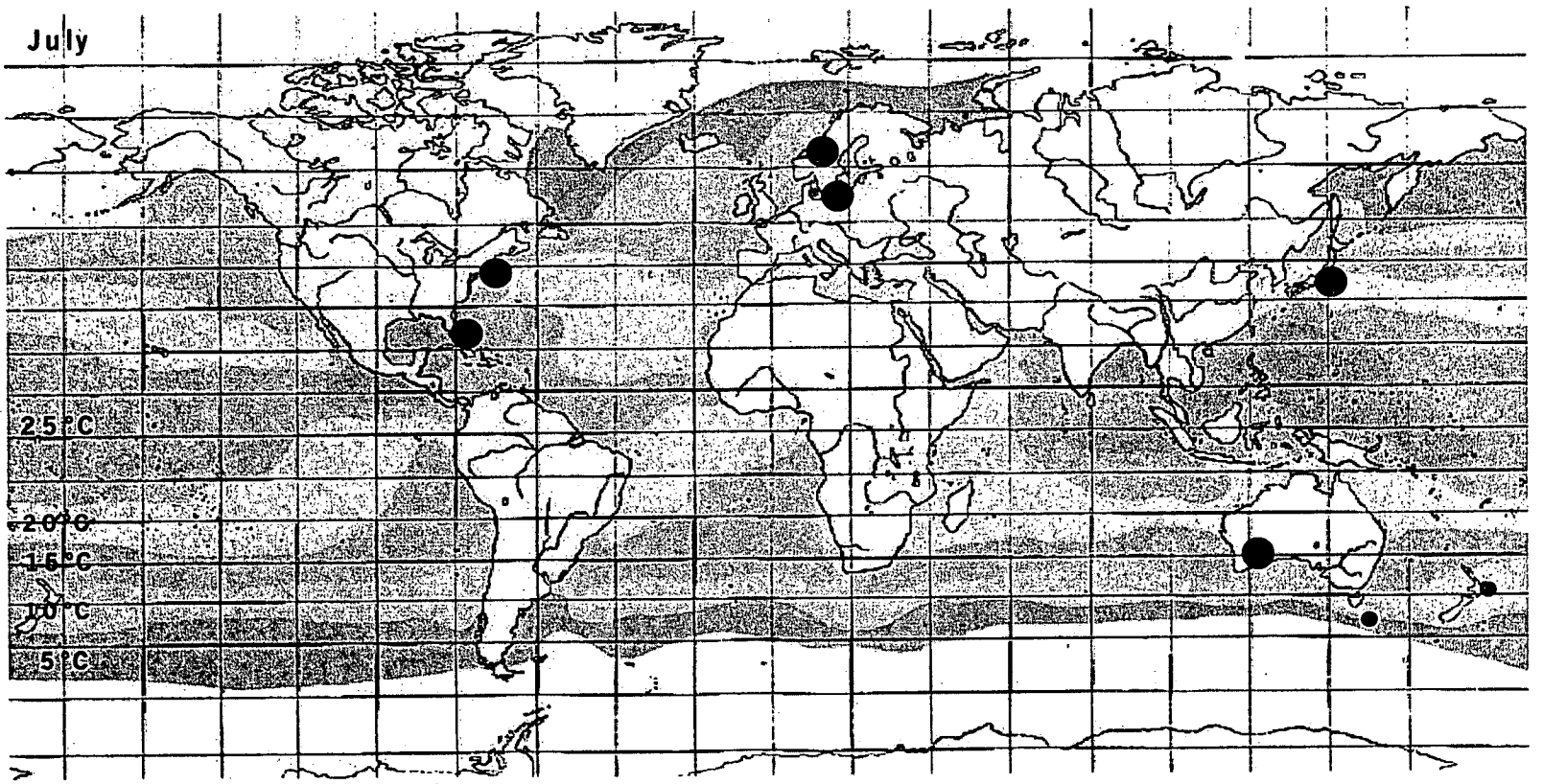


Figure 4.7; Global distribution of *Fibrocapsa japonica*. Large circle denotes bloom, small circle denotes sighting

P. Hargraves pers. comm., Florida Bay < 32 psu salinity; Tomas 1998). The blooms often occur in coastal and estuarine systems, and have been associated with El Niño climatic conditions over the summer months (Rhodes *et al.* 1993). *Fibrocapsa* blooms in Western Australia are often associated with autumn rainfall after dry summers, most likely due to an influx of nutrients (Colgrave pers comm.).

4.7 *Olisthodiscus luteus*

4.7.1 Global distribution

The distribution of *Olisthodiscus luteus* is confounded by past mis-identifications as *Heterosigma akashiwo* (=*carterae*) (see Chapter 2 for details). Hallegraeff and Hara (1995) reported a distribution of this benthic species in salt marshes of Europe, North America, South Africa and Japan.

4.8 *Haramonas* species

4.8.1 Global distribution

A new genus of raphidophytes, *Haramonas*, was coined by Horiguchi (1996), when he described the tropical species *Haramonas dimorpha*. This species thusfar has only been reported in Australian waters of Northern Queensland, around mangrove forests. There have been also sightings of a different *Haramonas* in Sydney Harbour, Australia, in 1996 and 1998 when it was associated with fish kills (P. Ajani pers. comm.). The species most likely is adapted to a estuarine environment (salinity 16 psu) and may be linked to terrestrial pollution. The identity, ecophysiology and toxicity of this species is currently under investigation.

4.9 Discussion

4.9.1 Factors affecting raphidophyte distribution

Raphidophytes have a known geographic distribution throughout temperate, subtropical and tropical waters. Distribution has been well documented in areas where the environment is regularly monitored such as Japan, USA, Canada, Norway, New Zealand and southern Australia. Often these monitoring sites are in areas of intensive aquaculture. However, examples do exist of raphidophytes species being present in brackish shallow lagoon areas (Patos Lagoon: Odebrecht, 1995), salt lakes (Selton Inland Sea, USA: Tiffany 1998) and seasonally closed water bodies such as Wilsons Inlet, Western Australia. Studies of *Chattonella* blooms in Japan demonstrate a relationship between shallow and stratified water bodies and events causing mixing of the water column as shown in Table 4.5. Most records indicate that Japanese raphidophytes are restricted to eutrophic waters.

Raphidophyte algae may only bloom under certain stimuli such as a temperature increase, salinity gradient or introduction of an apparent (micro) nutrient in the water column. Hence, alga such as *Chattonella* may exist in many locations for the majority of the year as part of the hidden flora. Imai (1998) reported *Chattonella* cells in Japan occur in background levels (< 100 cells per litre) for many months of the year or as part of a benthic cyst bed. No evidence of a *Chattonella* cyst bed has been found in Australian waters, even in areas such as Boston Bay where large blooms have been reported (Bolch and Hallegraeff unpublished).

Table 4.5. Reported environmental conditions proceeding *Chattonella* blooms

Location	Depth	Previous conditions	Nutrients	Bloom	Authority
Chattonella marina					
Harima-Nada	40m	Possible stratification Heavy rains-Jul 15-21	High Si Very high PO_4 Very high Fe Low NO_3 & NO_2, Low NH_4	July 21 Low salinity	Montanie *et al.* 1989
Port Lincoln	20m	Strong wind event. Sediment suspension	Unknown	April 17 High fish mortalities	Clarke 1996
Esperanza Inlet. British Columia	120m	Heavy rain storm previous to loom	Unknown	Sept. High salmon mortalties	Nicki Haigh pers comm..
Chattonella antiqua					
Harima-Nada	40m	Possible stratification	Influx of nutrient rich water from Naruto Channel	Yes	Fukase 1980
Seto Inland Sea		Stratification 20 Jul – 3 Aug then wind mixed	0-5m low N & P 10-20m high N & P	7-9 Aug	Nakamura *et al.* 1989
Harima-Nada	40m	Strong winds		Yes	Yanagi 1989

Incomplete records of raphidophyte blooms may occur due to (i) avoidance by wild fish populations reducing or eliminating any fish mortalities (ii) tolerance of blooms by fish or (iii) blooms occurring in uninhabited locations (particularly for Australia). Studies by Kobayashi and Ishida (1984) have demonstrated that yellowtail (*Seriola quinqueradiata*) display an olfactory response to *Chattonella* cells at a density of less than 2 cell per ml (1000 cells per litre). Alternatively, sand whiting (*Sillaginodes punctatus*), an estuarine and coastal fish species did not display any distress response to the toxic raphidophyte *Chattonella marina*, or to the reactive oxygen species hydrogen peroxide (H_2O_2) at concentrations of 1000 ppm and may have a tolerance to the toxic principle involved (Marshall, Munday and Hallegraeff unpublished data). Leatherjacket fish (Family Monacanthidae) have also been observed to be tolerant to *Chattonella* blooms in the Seto Inland Sea. Farmed finfish such as salmonids and yellowtail often behave as a sensitive bio-assay for the presence of toxic raphidophyte algae through changes in fish behaviour.

The present review demonstrates that raphidophytes exist outside of waters associated with aquaculture, and therefore most likely have a wider distribution than has been reported in the literature. The finfish aquaculture industry is expanding globally, resulting in an increased awareness of harmful raphidophyte blooms.

There has been a nearly a 4 fold increase in harmful raphidophyte blooms since 1990 (Fig 4.8), which may be contributed to increased education in conjunction with aquaculture operations as well as the advent of algal monitoring programs in may areas. Similarly, there has also been a rapid increase in the number of countries reporting blooms, from only 10 countries reporting blooms up to 1987, to 27 countries having reported raphidophyte blooms by 2000.

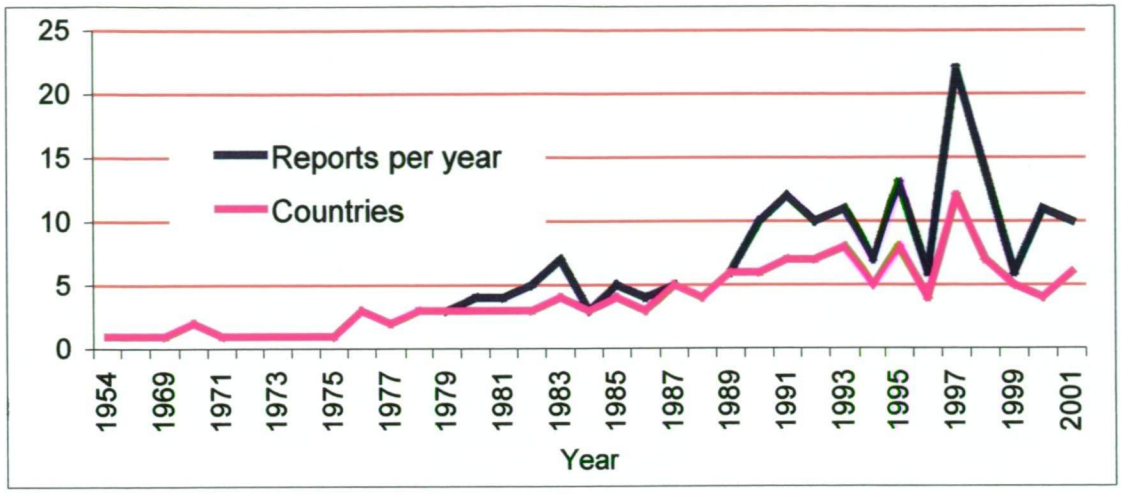


Fig 4.8, The annual increase in reports and number of countries reporting raphidophytes over the last 50 years.\

Chattonella spp. and *Heterosigma* are the predominant species of all reported sightings (Fig 4.9). There have been yearly sightings of *C. marina* and *C.antiqua* in the Seto Inland Sea since 1967. The majority of the research into *Chattonella* spp. taxonomy, ecology, toxicity and physiology has been reported by Japanese researchers. Similarly, there have been yearly sightings of *Heterosigma akashiwo* after salmon mortalities occurred in British Columbia, Canada and Washington, USA in 1976 (Horner 1997) with a large proportion of *Heterosigma* research concentrated in British Columbia (Canada) and Puget Sounds (USA) in the last decade.

Present trends in raphidophyte distribution indicate that *Chattonella* spp. and *Heterosigma akashiwo* will be considered to have a cosmopolitan distribution, rather than being only associated with finfish aquaculture and eutrophication.

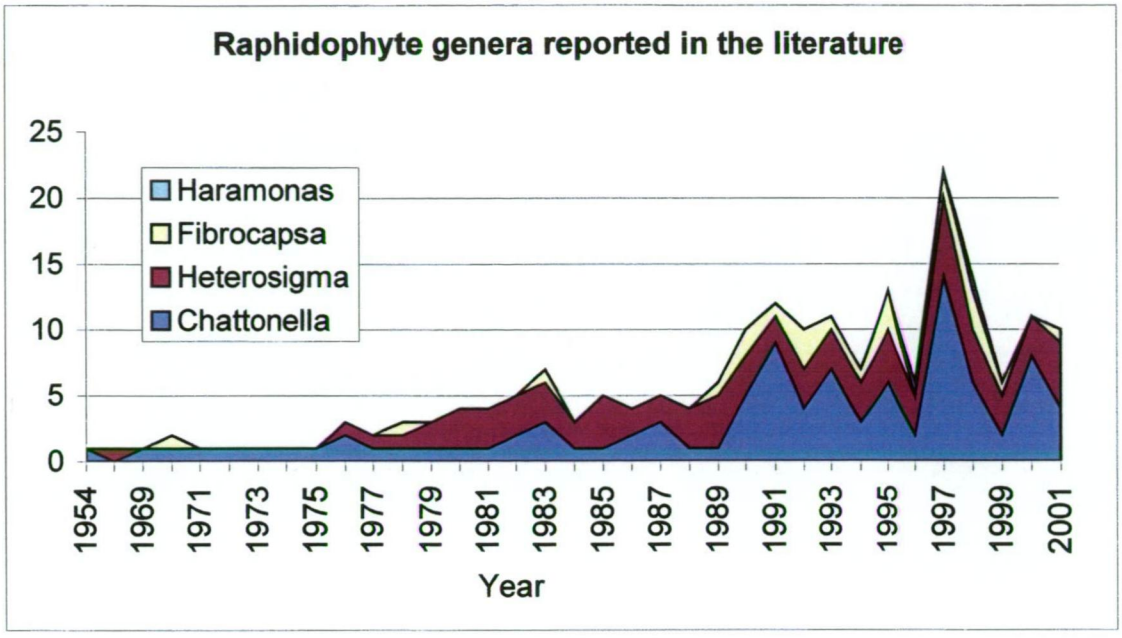


Fig 4.9. Diversity of raphidophyte species reported in the literature from 1954.

4.9.2 Validity of Identifications

Questions may be raised over the validity of the distribution of some raphidophytes presented in the literature, due to the lack of verification of identification. Without investigation by Transmission Electron Microscopy (TEM), it is often not possible to determine the identification of the closely related *Chattonella* species. Vrieling *et al.* (1995) reported among *C. marina* on the Dutch coast "a larger, slightly flattened and slender tail-bearing species, which resembled *C. antiqua*". Although light microscopy photographs are presented, the cells are not significantly different to *C. marina* in both size and morphology to be able to determine whether a second species was present or not. According to Vrieling *et al.* (1995), immunofluoresence-assay for *C. antiqua* was very weak or no labeling was observed for obovoid cells using the AT-86 monoclonal antibody specific. There is little evidence that the AT-86 antibody is specific in distinguishing North European species of *Chattonella* and may not be applicable to confirm the presence of *C. antiqua* cells in the Dutch Wadden Sea.

Reports of *C. antiqua* in Florida (Tomas 1998) and Brazil (Odebrecht and Abreu 1995) also remain unverified, other than by light microscopy. These blooms co-occurred with *C. marina*, and it is possible that mis-identification of unusual phenotypes of *C. marina* occurred. These two species differ in size but are similar in shape, hence it is difficult to distinguish between small *C. antiqua* and large *C. marina* cells. A range of morphotypes of *C. marina* has been noted in the present work, as shown in Chapter 1 (Plate 1.1). All other sightings of *C. antiqua* occur in the Asia-Pacific region, namely Japan and Korea suggesting a localized distribution. The separation of the *Chattonella* is further discussed in Chapters 2 and 3.

The morphology of *C. subsalsa* is very similar to *C. marina*, such that the taxonomy of this species is under question (Hallegraeff and Hara 1995). The type species of *C. subsalsa* needs to be re-examined to determine whether it is a separate species or a sub-species of *C. marina* (see Chapter 2). It is possible, that like *C. antiqua*, *C. subsalsa* has a very restricted distribution within the Western Mediterranean Sea and that other sightings are mis-identifications. Reports of *C. subsalsa* in Barra Lagoon, Brazil (Domingos and Menezes 1998) may be morphotypes of *C. marina*, and warrant further investigation.

Morphology of the various life stages of *Chattonella spp.* has not been sufficiently examined. Small cells such as those identified as *Heterosigma akashiwo* in a *Chattonella* bloom may be gamete cells of *Chattonella*, raising questions as to the validity of some identifications from co-occurring blooms.

The 1998 bloom of *Chattonella verruculosa* on the Danish / Norwegian coasts triggered debate on whether raphidophyte species are part of the cryptic native flora or introduced through some transport vector. Nehring (1998) claimed *Chattonella antiqua*, *C. marina*, *Fibrocapsa japonica* and *Heterosigma akashiwo* to be non-indigenous in northern Europe due to the lack of historical records of these species. Incomplete taxonomic identification of raphidophyte species through poor preservation of material, particularly after long term storage, must

be considered when addressing historical data. Nutrient cycles and climate-induced change have been suggested as possible vectors to enhance the survivability of raphidophytes (Nehring 1998). Anthropogenic based nutrient enrichment (eutrophication) and the rapid increase in aquaculture are likely to have resulted in increased reports of raphidophytes and other harmful algal blooms world wide. It is unlikely that raphidophyte species would survive transport through ballast water exchange in the vegetative state due to the fragile nature of the cells.

There needs to be careful examination of raphidophyte cells before identifications are reported. The restricted knowledge of life cycles and ecophysiology also confounds the problem of identification in this problematic group of algae. Stimuli for bloom initiation have often been circumstantially associated with eutrophic water bodies, commonly with finfish aquaculture. Micro/macronutrient and physical triggers for raphidophyte blooms need to be addressed before prediction and management of this algal group can be achieved.

4.10 References

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Chapter 5

Comparative ecophysiology of the harmful alga *Chattonella marina* (Raphidophyceae) from South Australian and Japanese waters[1]

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5.1 Abstract

The raphidophyte flagellate *Chattonella marina* was successfully cultured from Boston Bay (South Australia), coincident with mass mortality of farmed bluefin tuna (*Thunnus maccoyii*) in April 1996 . Grown under laboratory conditions at 150 µmol $m^{-2}s^{-1}$ irradiance, optimal growth (>0.5 d^{-1}) occurred at 25°C temperature, 30 psu salinity, but good growth (>0.3 d^{-1}) also occurred between 10 and 30 °C temperature and 15 to 45 psu salinity. However, cultures grew much faster at 450 µmol $m^{-2}s^{-1}$ irradiance (1.08 d^{-1}). While Australian *C. marina* had similar temperature and salinity requirements as well-studied Japanese cultures from the Seto Inland Sea, the Australian strains exhibited a light saturation level for growth 4 times higher than reported from Japan (150 µmol $m^{-2}s^{-1}$). An adaptation to higher light intensities was reflected in higher concentrations of mycosporine-like amino acids (MAA's) in the Australian strains. The different light adaptation phenotypes were still apparent after long term culturing under similar physiological conditions. Potential growth habitats for this ichthyotoxic flagellate in the Australian region and implications for finfish aquaculture industries are discussed.

5.2 Introduction

The raphidophyte flagellate *Chattonella marina* (Subrahmanyan) Hara et Chihara is a well known causative organism of mass mortalities of cultured fish in Japan, resulting in ¥7,100 million (US$0.5 billion) damage in 1972 alone (Okaichi, 1997).

In April 1996 a mass mortality of caged bluefin tuna (*Thunnus maccoyii*) in South Australian waters costing over Aus $45 million co-occurred with an algal bloom of *C. marina* at densities of 66,000 cells per litre (Hallegraeff *et al.*, 1998, Munday and Hallegraeff, 1998) . The potential threat of *C. marina* to the South Australian aquaculture industry has been questioned, however, on the basis that

bloom conditions of this species in Japan were not comparable with the Australian hydrological situation (Clarke, 1996).

The environmental requirements for growth of potentially harmful algae need to be understood to determine the conditions for formation and maintenance of blooms. Physical parameters such as temperature, salinity, irradiance, and water column stability can be used as an important management tool for predicting algal blooms in areas of intensive fish culture. The effect of temperature, salinity and irradiance on growth is also reflected in the motility and morphology of cells. Poor growth is often associated with the appearance of non-motile raphidophyte cells (see Tomas, 1978 for *Heterosigma akashiwo*, Khan *et al.*,1995b for *Chattonella antiqua*) . At present, the ecophysiology of *Chattonella* has not been studied outside Japan.

The raphidophyte *C. marina* produces poorly characterised neurotoxic, haemolytic and haemoglutinating compounds (Ahmed *et al.*, 1995; Khan *et al.*, 1996; Onoue and Nozawa, 1989; Onoue *et al.*, 1990) as well as superoxide and hydroxyl radicals that cause oedema formation in the gill lamellae, resulting in suffocation (Oda *et al.*,1992) . Blooms of this organism have also been linked to mass mortality of marine life on the Malabar Coast, India (reported as *Hornellia marina* by Subramanyan, 1954), and this species has also been reported to bloom in China (Tseng *et al.*, 1993) and Florida (Tomas, 1998).

The present study aimed to (i) determine the role of temperature, salinity and light in bloom formation of the Australian strain of *C. marina* ; (ii) assess if the Australian strain is physiologically similar to Japanese strains in order to decide whether the extensive work done in Japan can be applied to understanding Australian blooms.

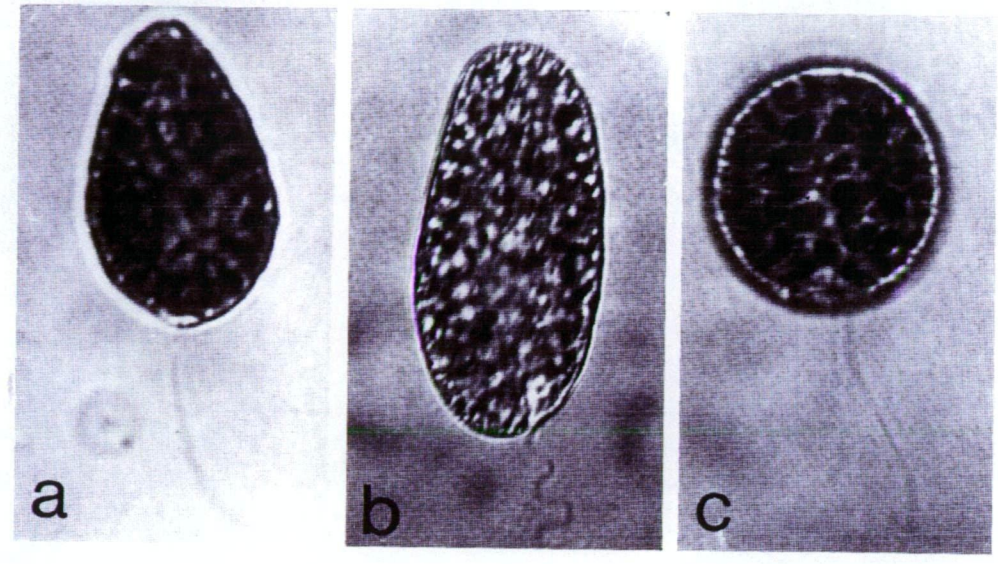


Figure 5.1. Light micrographs of *Chattonella marina* from South Australia showing three morphotypes (a) spindle, (b) oval (c) spherical morphology.

5.3 Method

5.3.1 Stock Cultures

A non-axenic clonal culture of *C. marina* (CMPL01) was isolated from Port Lincoln, South Australia, during April 1996. A Japanese strain of *C. marina* (N-118) was obtained for comparative studies from the NIES Collection, Japan (Origin:Seto Inland Sea/ Isolator:Yoshimatsu 1983). The cultures were maintained in GSe medium (Blackburn *et al.*, 1989) at 28 psu and 120 μmol $m^{-2}s^{-1}$ cool white light (Sylvania daylight fluorescent tubes). Culture media was prepared using southern Tasmanian seawater (Pirates Bay), aged in the dark at 5°C, as a base. Both seawater and nutrients were filter-sterilised through a 0.22μm filter (Gelman Sciences Supor membrane) into autoclaved culture vessels. Seawater of different salinities was prepared by either rotary evaporation or dilution using micro-filtered water (Continental Water Systems). Salinity was measured using a WTW LF 320 probe following the Practical Salinity Scale of 1978 (PSS 78).

5.3.2 Temperature and salinity

Cultures were pre-adapted to experimental conditions for a minimum of 8 generations (Thompson *et al.*, 1991) . A 1 ml aliquot of culture was inoculated into quadruplicate test- tubes containing 35 ml of GSe medium at 9 different salinities (10, 15, 20, 25, 30, 35,40, 45, and 50 ± 1 psu) and incubated at six temperatures (10, 15, 20, 25, 30, and 35 ±1.0°C) for up to 20 days. All salinities were tested at all temperatures in a cross factorial design.

Illumination was provided by 18 Watt daylight fluorescent tubes (Sylvania cool white) at 150 µmol $m^{-2}s^{-1}$ on a 12:12 light:dark cycle. Cultures of 130 ml volume were grown in 250 ml Ehrlenmeyer side arm flasks for each experimental condition and sampled every 2-3 days for observations of morphology and cell number using a Sedgwick Rafter cell. A minimum of 100 cells per sample were examined for morphology and motility and a minimum of 400 cells where possible were used to determine cell concentrations. Motility and morphology of cells were classified as motile or non-motile and spherical, oval or spindle shaped, as described by Khan *et al.* (1995a) (Figure 5.1). No differences in growth rates were detected between 250 ml Ehrlenmeyer side arm flasks and 35 ml test tubes.

5.3.3. Light experiments

Irradiance was measured using a spherical (4π collector) immersible quantum light meter (Biospherical Instruments, San Diego, USA. model QSL-100). Cells were pre-conditioned to irradiances of 10, 25, 50, 100, 200, 300, 400, 600, 800, 1000 and 12000 µmol $m^{-2}s^{-1}$ for a minimum of 8 generations prior to the experiment. Three 250 ml side arm flasks containing 150 ml of GSe media were inoculated with 3 ml for each treatment.

Table 5.1. Summary of two-way analysis of variance of growth rate as a function of temperature and salinity.

Source of variation	D.F.	Sum of squares	Mean square	F
Temperature	9	0.286	0.036	<0.001
Salinity	5	0.325	0.065	<0.001
Error	39	0.114	0.003	
Total	52	0.721		

5.3.4 Fluorometry

Growth in all experiments was measured by *in vivo* fluorescence using a Turner Design fluorometer (Model 10-AU) at 2-3 day intervals. Cultures were gently mixed using a Vortex mixer prior to measurement and sampling. Each set of experimental conditions included a blank of GSe medium to estimate background fluorescence. Calibration of fluorescence against cell numbers was based on a minimum count of 400 cells where possible.

5.3.5 Spectrophotometry

A 5 ml aliquot of culture in exponential phase for each light treatment was filtered on to 13 mm Whatman GF/F paper and stored at -70°C until processed. Pigments were extracted in 1 ml of methanol according to Jeffrey *et. al.* (1997) and scanned in a GBC UV Vis 916 spectrophotometer at 250-700nm.

5.3.6 Statistical analysis

Maximum culture growth rate (k) was calculated during the exponential growth phase using:

$$k = (ln\ N_t - lnN_0) / t$$

where N_t and N_0 are cell concentrations at time t and 0 (in days), respectively.

Divisions per day were calculated as:

$$k' = k / \ln 2$$

For temperature/salinity treatments, growth rates (k') for each treatment were combined by multiple regression. Curves were fitted using means of squares. Samples were analysed by a third order polynomial on temperature and salinity, with lower order interactions fitted to the data:

$$k' \text{ predicted} = 0.5578\ (\pm 0.238) - 0.1659\ (\pm 0.025)\ T + 0.06471\ (\pm 0.016)\ S + 0.0088\ (\pm 0.001)\ T^2 + 0.0017\ (\pm 0.0004)\ S^2 + 0.0002\ (\pm 0.0006)\ TS - 0.0001\ (\pm 1.62E^{-5})\ T^3 - 1.293E^{-5}\ (\pm 0.1.00E^{-5})\ T^2S + 2.6105E^{-6}\ (6.62E^{-6})\ TS^2 + 1.306E^{-5}\ (4.45E^{-6})\ S^3$$

A double exponential curve was fitted to the irradiance data.

5.4.1 Results

5.4.1 Effect of temperature and salinity on growth

For cultures grown at 150 μmol $m^{-2}s^{-1}$ irradiance, growth was maximal (0.47 d^{-1}) at 25°C and 30 psu salinity (Figure 5.2), with the highest cell numbers (5.0 x 10^7 cells per litre) being achieved on day 20. The dynamics of the growth curves (Figure 3a) differed with varying temperatures. Cultures grown at 10°C showed a longer lag phase (4 days) compared to cultures at optimal conditions (20-25°C) which had short lag (<2 days) and exponential phases (10 days). Cultures at higher temperatures (30°C) showed longer exponential phase (12-14 days) and lower growth (0.27 d^{-1} at 30°C compared to 0.33 d^{-1} at 25°C). Statistical analysis showed significant differences ($p<0.05$) in growth rate of *C. marina* over the temperature-salinity matrix (Table 5.1).The variances of means for

growthwere not significant, shown as subscripts in Figure 5.2, except for temperatures above 30°C and at extreme salinities (<10 psu, >50 psu). Good growth (>0.3 d^{-1}) occurred over a wide range of salinities (20 to 35 psu) and temperatures of 20 to 30 °C. Growth occurred at 50 psu, but only at temperatures 15-25°C. There was no growth below 15 psu. ANOVA showed that the polynomial response model (Figure 4) was highly significant ($P< 0.001$) with around 60% of the data fitting the variables ($r^2 = 0.587$). The model yielded greatest growth rates (>0.4 d^{-1}) at temperatures between 20° and 29°C and salinities of 20-37 psu.

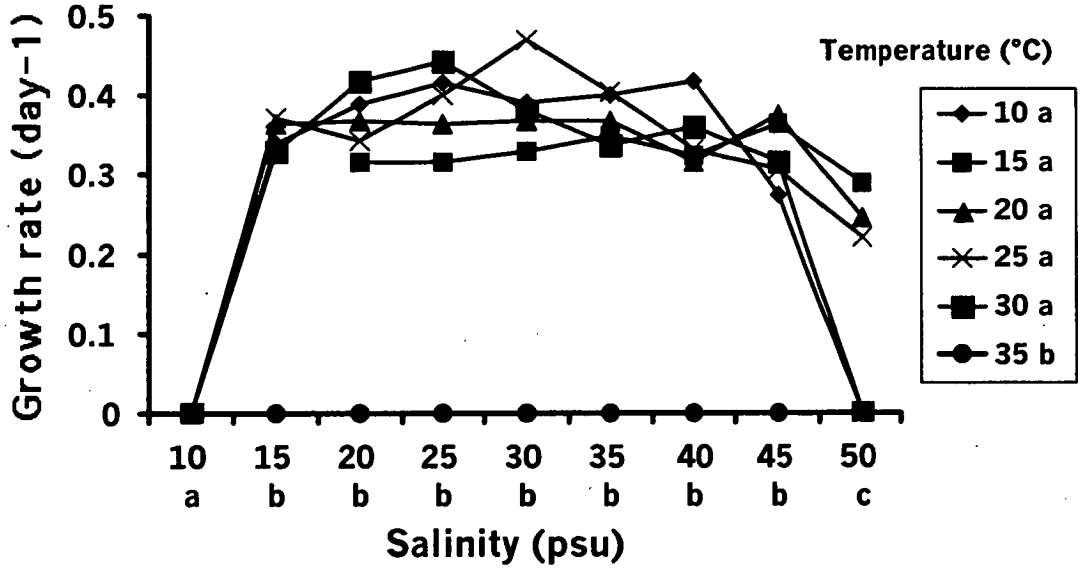


Figure 5.2. Growth rate of the South Australian *Chattonella marina* (SE < 0.07) as a function of temperature and salinity at constant light (150 µmol $m^{-2}s^{-1}$). Subscripts (*a-c*) denote statistical differences in growth rates for each treatment ($p<0.05$).

Figure 5.3a

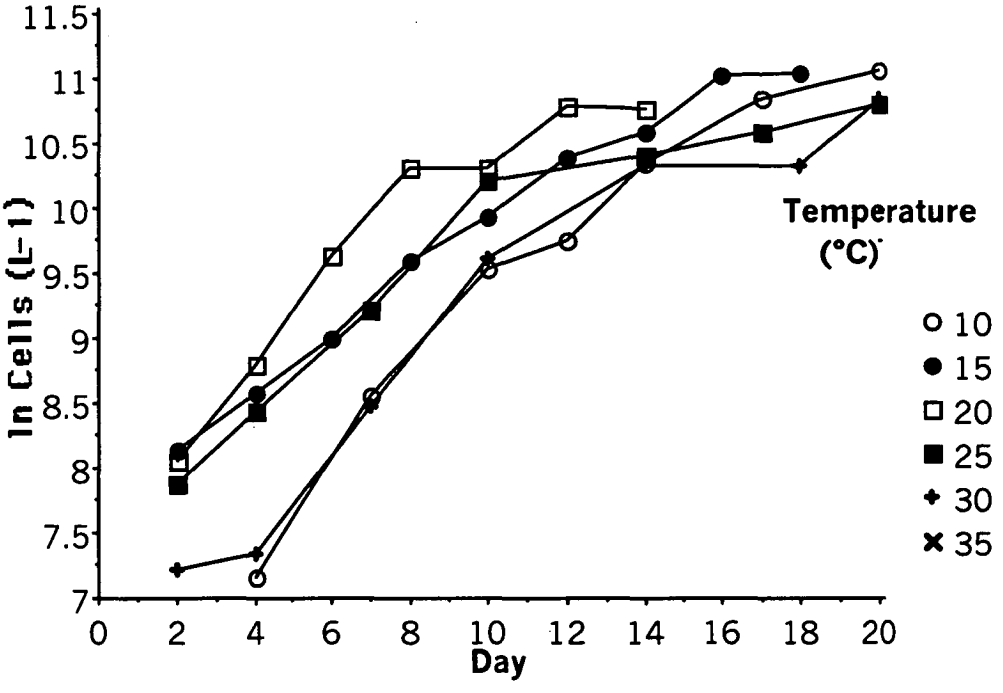


Figure 5.3b

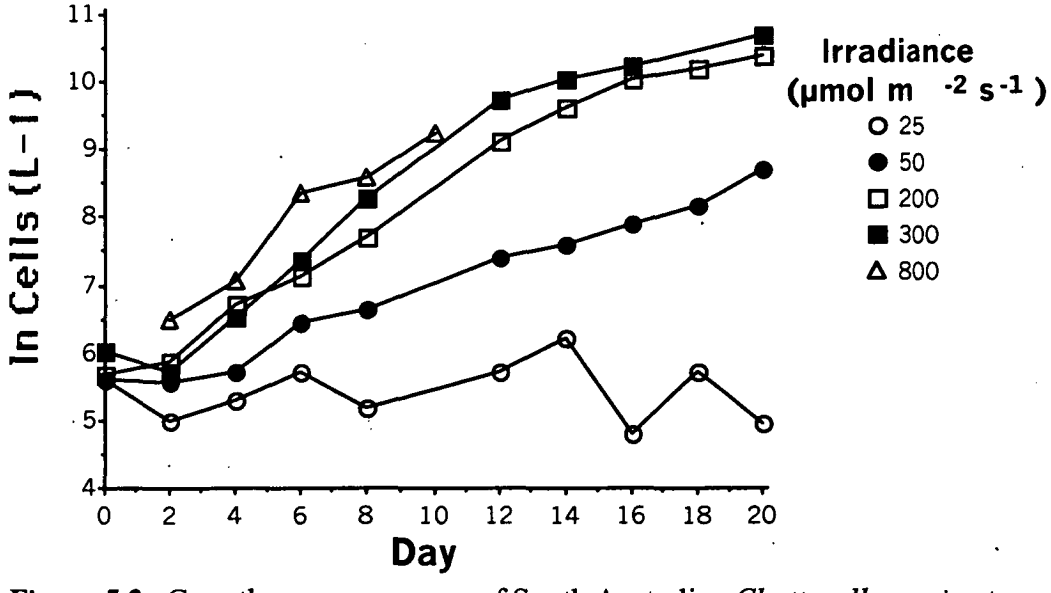


Figure 5.3. Growth curve responses of South Australian *Chattonella marina* to (a) different temperatures at constant salinity and irradiance (25 psu, 150 µmol $m^{-2}s^{-1}$) and (b) different irradiances at constant temperature and salinity (25 °C , 28 psu).

5.4.2 Effect of Irradiance on growth

The dynamics of the growth curve varied with irradiance (Figure 5.3b). For the Australian strain, low irradiance (25 μmol $m^{-2}s^{-1}$) produced a long lag phase, and exponential growth was not achieved during a 20 day time period. The duration of lag and exponential phases decreased with increasing irradiances,

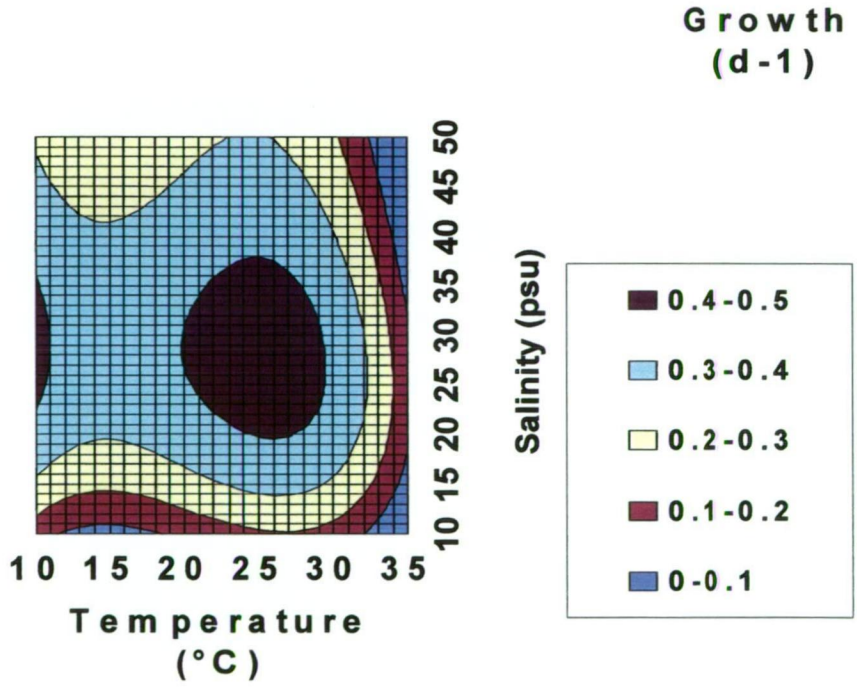


Figure 5.4. Contour plot of the third order polynomial model showing the effect of temperature and salinity on the growth rate of the South Australian *Chattonella marina*.

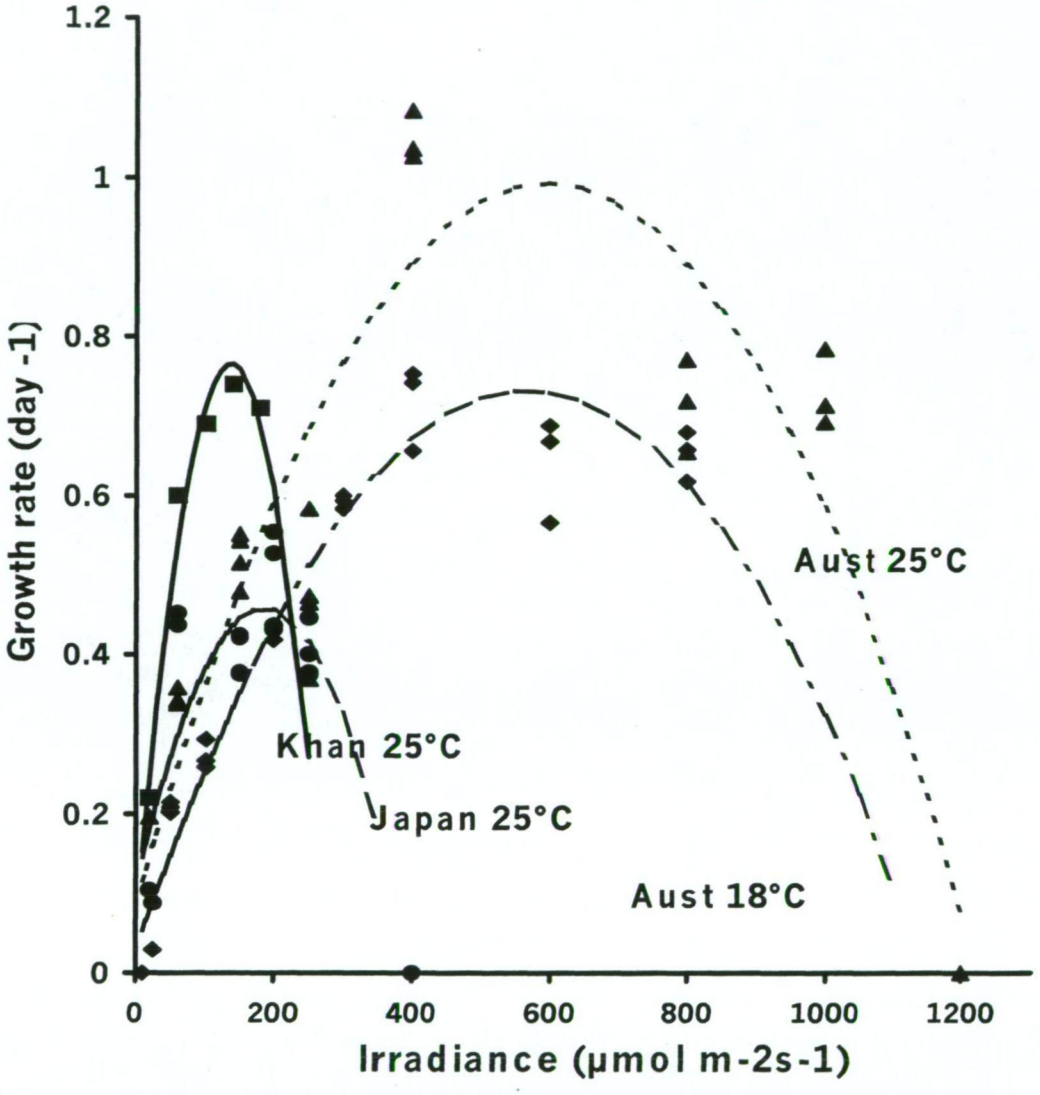


Figure 5.5. Growth rate of *C. marina* (d^{-1}) as a function of irradiance fitted with a double exponential curve. Australian strain (CMPL01) at 18° and 25°C temperature and 25 psu salinity (present study); Japanese strain (NEIS-118) at 25°C temperature and 25 psu salinity (present study); and Japanese strain at 25°C temperature and 30 psu salinity (adapted from Khan,1998).

with high irradiances (300 μmol $m^{-2}s^{-1}$) resulting in short exponential phases (10 days). Cultures showed exponential growth at all irradiances except at 10 μmol $m^{-2}s^{-1}$(negative growth), 25 μmol $m^{-2}s^{-1}$ (zero growth) and 1200 μmol $m^{-2}s^{-1}$ (negative growth). Light saturation with the Australian *C. marina* was observed at 400 μmol $m^{-2}s^{-1}$ (Figure 5.5), with slightly lower growth rates at 600 and 800 μmol $m^{-2}s^{-1}$, and photo-inhibition above 1000 μmol $m^{-2}s^{-1}$. The maximal growth rate of 1.08 d^{-1} was observed at 450 μmol $m^{-2}s^{-1}$.

Reduced growth rates at irradiances above 600 μmol $m^{-2}s^{-1}$ coincided with the appearance of presumed gametes (smaller cells in fusing pairs). Under the same experimental conditions, the Japanese strain (N-118) showed maximal growth (0.55 d^{-1}) at 200 μmol $m^{-2}s^{-1}$ with photo-inhibition occurring below 400 μmol $m^{-2}s^{-1}$

.5.4.3 *Cell morphology*

Under optimal conditions (25°C, 30 psu) cell morphology in exponential phase and early stationary phase was dominated by the spindle form (82-96%), and in lag phase the spherical cell shape (51%). A decline in oval cell shapes was observed at day 20, indicative of the onset of stationary phase. Under sub-optimal conditions, cell morphology was influenced by both temperature and salinity. The effect of temperature on morphology was minimal, with very little variation apparent in the exponential stage under the temperatures tested. Spindle morphology was prevalent at the lower irradiances in exponential phase. Poor cell morphology (spherical cells) became more apparent with increasing irradiances (> 100 μmol $m^{-2}s^{-1}$).

5.4.4 Cell motility

Cell motility was affected by growth phase, with more motile cells occurring in the exponential phase than the lag or stationary phase. There was a decrease in the percentage of motile cells from 70 ± 7 % in exponential phase at the higher (>45 psu; 39 ± 6% motile cells) and lower (<15 psu; 0% motile cells) salinities. Temperature did not have an effect on cell motility except at the extreme range of growth conditions (35°C). Cell motility was also poor (24 ± 4%) at low irradiance (25 μmol $m^{-2}s^{-1}$) and during early exponential phase when compared to mid range irradiances (50-200 μmol $m^{-2}s^{-1}$; 63 ± 11 % motile cells). Motility for high irradiances (above 400 μmol $m^{-2}s^{-1}$) was high in exponential stage but decreased rapidly to 14 % once cells entered stationary phase (day 14).

5.4.5 Spectrophotometric Pigment Analysis

The absorbance spectra (250 - 700 nm) for *Chattonella marina* showed considerable variation between Australian and Japanese strains and with irradiance acclimation (Figure 5.7). In cultures acclimated to an irradiance of 300 μmol $m^{-2}s^{-1}$, the absorption peaks for chlorophyll *a* and associated accessory pigments (430-450nm, 665 nm) were similar. However, the level of mycosporine-like amino acids (MAAs) in the Australian strain differed from that of the Japanese strain ($A_{320-340}$:A_{665} = 1.76 for Australian strain, 1.24 for Japanese strain). The Australian culture acclimated to very high irradiance (900 μmol $m^{-2}s^{-1}$) contained high levels of MAAs with $A_{320-340}$:A_{665} a ration of 9.77.

5.5 Discussion

5.5.1 Temperature and salinity

In laboratory cultures, the South Australian strain of *C. marina* showed a wide temperature-salinity tolerance. Maximum growth rates were achieved at 25°C, 30 psu, while good growth rates occurred from 10 to 30°C temperature and 15 to 45 psu salinity. Bloom conditions under which the organism was originally isolated were 17.0 to 17.9°C temperature and 35.3 to 36.9 psu salinity. Maximum cell concentrations achieved in culture at 15°C of 8.6×10^7 cells per litre were 3 orders of magnitude higher than found at Boston Bay in April 1996 (6.6×10^4 cells per litre; Clarke, 1996).

Table 5.2. Conditions for growth of *Chattonella* species reported in the literature, compared to the present study.

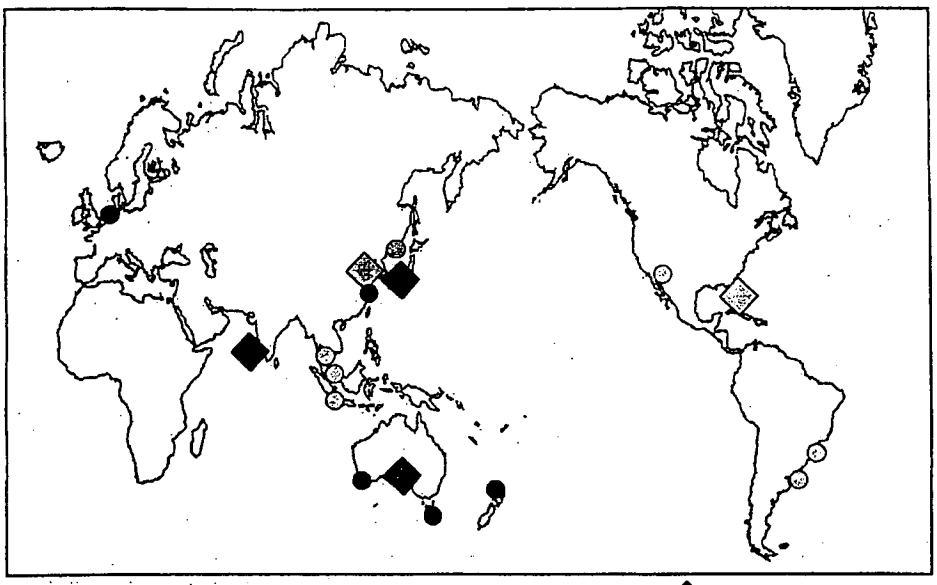
Temp (°C)	Salinity (psu)	Irradiance (μmol $m^{-2}s^{-1}$)	Growth rate (d^{-1})	Authority
Chattonella marina				
15-30	10-35	110	0.81	Yamaguchi *et al.*, 1991
13-30	-	-	-	Yamochi,1984
25	20-30	120	0.60	Khan *et al.*,1998
15-30	**15-50**	**150**	**0.47**	**Present study**
25	**28**	**400**	**1.08**	**Present study**
Chattonella antiqua				
15-30	10-35	110	0.97	Yamaguchi *et al.*, 1991
10-30	15-45	140	0.51	Nakamura & Watanabe, 1993
11-30	-	-	-	Yamochi, 1984
20	33	-	0.45-0.67	Kohata & Watanabe,1988
25	25-35	180	0.65-0.71	Khan *et al.*,1996
Chattonella verruculosa				
11-20	15-35	150	1.74	Yamaguchi *et al.*, 1997

30 °C temperature (M. A. Tiffany, pers. comm.) *Heterosigma akashiwo* has tolerance ranges of <2 to >30°C temperature and 1 to >50 psu salinity (Smayda, 1998) . Ranges for other *Chattonella* spp. are listed in Table 2.

The present study demonstrates that *C. marina* could potentially form a dense bloom under the salinity, temperature and light conditions prevailing in South Australian waters. Unfortunately, there exists no local coastal monitoring data for Boston Bay to compare natural abundances under optimal or sub-optimal conditions. Possible favourable habitats for this organism in Australia would range from Sydney to Perth, with growth only restricted in areas of temperatures below 10°C and above 30°C. Water temperatures indicate that this species could over-winter in South Australian conditions. This temperature and salinity range is comparable to that required by Japanese strains of *C. marina* (Table 5.2). Japanese strains of *C. marina* show maximum growth at temperatures of 25° and salinities of 25 psu with broad sub-optimal tolerances towards temperature of 15-30°C and salinities of 15-35 psu (Yamaguchi*et al.*, 1991; Khan *et al.*, 1998). Environmental studies in Japan show that *C. marina* produces resting cysts at temperatures below 15°C (Imai and Itoh, 1987), but only very few *Chattonella* cysts have thus far been recovered from South Australian waters (Hallegraeff & Bolch, unpublished).

The dynamics of the growth curve for the Australian *C. marina* also compare favourably with reports by Yamaguchi *et al.* (1991) on a Japanese strain, with increased duration of lag phase at lower temperatures (15°C) and short exponential phases (6-10 days) at optimal growth conditions. Growth rates for the Australian strain at 150 µmol $m^{-2}s^{-1}$ irradiance were highest at 25°C temperature and 30 psu salinity (0.47 d^{-1}), but the species also grew consistantly (> 0.2 d^{-1}) over a wide range of salinities and temperatures (10-30°C, 15-40 psu), indicating the euryhaline and eurythermal nature of this species, typical of a coastal organism (Tomas, 1978) . Similar wide salinity tolerances have been found for other Raphidophyte species. *Chattonella cf. subsalsa* has been recorded to bloom in the Salton Sea (California, USA) in 50 psu salinity waters. Blooms of *C. marina* have been reported from India (Subramanyan,1954), China (Tseng *et al.*, 1993) and Florida (Tomas, 1998) but the species also occurs in New Zealand (Rhodes, pers comm.), Korea (Park, 1991), Brasil (Odebrecht and

Abreu, 1995) and the Netherlands (Vrieling *et al.* , 1995). The global distribution of *C. marina*, summarised in Figure 5.6, reflects the broad environmental tolerance of this species. However, in the light of the results of the present study, we suggest the existence of 2 or possibly 3 eco-phenotypes, with strains from Asia (Japan/Korea/China) and Australia/New Zealand having similar temperature requirements but different light requirements, and a third group inhabiting tropical waters (India and possibly Florida). Physiologically different strains have also been reported for a related species, *Heterosigma akashiwo* (Watanabe *et al.*,1982). *C. marina* has not been known to bloom previously in Australian



Global distribution of *Chattonella marina*; ◆ blooms associated with fish mortalities; ◈ blooms not associated with fish mortalities ; ● confirmed occurrences ; ◍ species identity to be verified

Figure 5.6. Known global distribution of *Chattonella marina*.

waters, although low density occurrences have been noted in some southern Australian estuaries, including the Derwent estuary, Tasmania (Jameson & Hallegraeff, unpublished data) and Nornalup, Western Australia (Hosja, pers comm.).

Preliminary comparative molecular genetic studies between Japanese and Australian *C. marina* have thus far failed to detect any differences in the large subunit ribosomal RNA and ITS regions (J. Tyrrell, unpublished)

An ANOVA analysis of means of squares (Table 5.1) for both temperature and salinity showed that there is a strong effect on growth exercised by salinity. This is also supported by the variation in morphology and motility over the range of salinities. The weaker temperature*growth interaction indicates that the Australian *C. marina* is capable of bloom formation in sub-optimal temperatures (18°C). Salinity as a controlling factor for growth is linked to the nature of coastal and estuarine ecosystems compared to predominantly temperature-controlled oceanic environments (Prakash, 1967).

5.5.2 Morphology and Motility

Maximum growth was reflected in spindle cell morphology and motility. Cell morphology was a good indicator of culture health, as previously shown in *H. akashiwo* (Tomas, 1978) and *C. antiqua* (Furuki *et al.*,1981; Nakamura and Watanabe, 1983; Khan *et al.*, 1995b) . Spherical cell morphology of *C. marina* was associated with low salinities, low and high irradiance, and lag phase, but not with temperature, as found in *C. antiqua* (Khan *et al.*, 1995b). The occurrence of spindle shaped cells over a wide temperature range related to the broad temperature tolerances of the Australian strain.

Salinity plays a major role in determining morphology of the cells, along with stage of the growth cycle. Temperature defined the limits of growth for this organism but did not have a major role in determining cell morphology, and hence cell viability, supporting that small variations in temperature may not be a

factor in bloom maintenance. However, temperature still can be a determining factor in bloom initiation through temperature dependant cyst germination (Imai and Itoh, 1987).

5.5.3 Irradiance

Optimal growth rates for the Australian *C. marina* at 150 μmol $m^{-2}s^{-1}$ (0.47 d^{-1}) observed in the temperature-salinity experiments were significantly lower than reported for Japanese strains (0.81 d^{-1}; Yamaguchi *et al.*,1991). We believe that this can be partly attributed to the different temperatures at which the experimental cultures were grown (Australia 18°C, Japan 25°C) as well as physiological differences in light requirements. The present experiments indicate that irradiance played a major role in division rate, with comparable growth rates of 0.46 d^{-1} being achieved in sub-optimal growth conditions (18°C, 25 psu) under irradiances 4 times as high as used in Japanese studies (400 μmol $m^{-2}s^{-1}$ compared to 110 μmol $m^{-2}s^{-1}$; Yamaguchi *et al.* ,1991). Under optimal conditions of 25°C temperature, 25 psu salinity and 400 μmol $m^{-2}s^{-1}$ irradiance, a growth rate of 1.08 d^{-1} was achieved for the Australian cultures. The difference in saturation level of light may indicate an ecological adaptation of *C. marina* to higher irradiances in the Australian environment. Boston Bay (South Australia) receives an average summer irradiance of 7.9 hours per day at 26.83 MJ/m^2 (Bureau of Meteorology, pers. comm.) with an average light penetration (Secchi disc) of 6-10 m, where as the Seto Inland Sea receives average irradiances 6.8 hours at 18.3 MJ/m^2 (Japanese Meteorological Agency) with penetration depths (Secchi disc) of less than 4m (Hashimoto *et al.*, 1997). This represents a difference of 18% sunshine hours per day and 50% irradiance for Australian conditions when compared to the Seto Inland Sea, Japan.

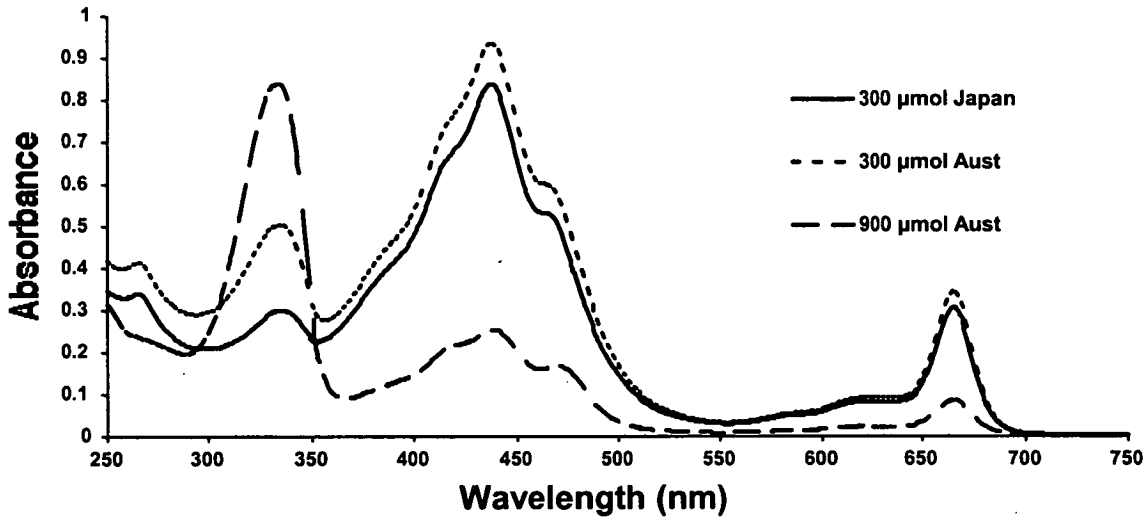


Figure 5.7. Absorbance spectra (250-700nm) of methanol extracted pigments from *Chattonella marina* . Peak absorbance occurring at 334, 438 and 665 nm.

An adaptation to the high irradiance of the South Australian environment is reflected in the greater production of photo-protective MAAs by the Australian strain when compared to the Japanese strain. Ratios of UV absorbance (320-340 nm) to chlorophyll *a* (665 nm) for *C. antiqua* were found to be 1.43 using tetrahydrofuran:methanol 20:80 as a solvent (Jeffrey *et al.* 1999). This value is similar for *C. marina* conditioned at 200 µmol $m^{-2}s^{-1}$ ($A_{320-340}:A_{665}$ = 1.76 Australian strain, 1.24 Japanese strain), but not at 900 µmol $m^{-2}s^{-1}$ ($A_{320-340}:A_{665}$ = 9.77 Australian strain). Similar adaptations have been noted in Antarctic species (Helbing *et al.*, 1996) and other marine organisms (Dunlap and Yamamoto, 1995; Neale *et al.*, 1998) as a protective mechanism against UVA and UVB associated damage. Work in progress will assess the photo-adaptive strategy of the Australian *C. marina* , including a possible effect of MAAs on reactive oxygen species production.

5.6 Conclusions

Temperature, salinity and light are important factors contributing to population growth of *C. marina*, but only partially define the bloom dynamics of this species. The Australian *C. marina* has a similar broad tolerance to temperature and salinity as the Japanese strains (Yamaguchi *et al.*, 1991; Yamochi, 1984) , but shows a significantly different response to irradiance. Irradiance may play a major role in bloom formation for the South Australian *C. marina* strain and to a much greater degree than for the Japanese strain. Accordingly, research done by Japanese workers may not be directly applicable to the Australian situation when considering management implications of *C. marina* blooms. Other factors such as stratification, nutrient assimilation, life cycle and vertical migration are the subject of continuing investigations and will ultimately be integrated into the development of a comprehensive bloom prediction model.

Acknowledgments

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Chapter 6

Differences in photoprotective pigment production between Japanese and Australian strains of *Chattonella marina* (Raphidophyceae)[1]

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6.1 Abstract

Previous studies have shown that isolates of *Chattonella marina* from Australia and Japan exhibit differences in tolerance to high intensities of visible light. Here we show that the Australian strain of *C. marina* produces around 5 times more UV-absorbing mycosporine amino acids (MAAs) than the Japanese strain. This corresponds with 66% increased growth by the Australian strain under UVB exposure compared to no UV exposure. The MAA mycosporine-glycine, which reportedly acts as an antioxidant, was found in high quantity (110 *fg* $cell^{-1}$) in the Australian but was absent in the Japanese strain. In contrast, changes in the concentration of violaxanthin and zeaxanthin per cell were 4.7 –4.8 times greater in the Japanese relative to the Australian strain suggesting that the Japanese strain uses a xanthophyll cylce to moderate inhibition by high PAR irradiance. Increased MAA production under high irradiance was also observed in other Australian strains of *Chattonella*, but not noted in other Japanese strains suggesting ecophenotypic adaptation due to differing environmental conditions

6.2 Introduction

Marine phytoplankton require photosynthetically active radiation (PAR: 400-750 nm) accompanied by ultraviolet radiation (UVR). However, a wealth of scientific evidence indicates that UVB (280-320 nm) radiation is damaging to organisms (UNEP 1989, 1991) while UVA (320-400 nm) may be inhibitory or involved in photorepair of UV-induced cell damage (Vincent and Roy 1993). Few plankton can sense UV-B and cells cannot detect an increase in UV-B when accompanied by other wavelengths (Roy 2000). One mechanism by which phytoplankton may respond to UV radiation is the synthesis of intra- or extracellular UV-absorbing compounds which may reduce UV radiation from reaching UV-sensitive targets such as DNA or photosynthetic reaction centers (Roy 2000). Mycosporine-like amino acids (MAAs) absorb between the range of 309-360 nm (Nakamura *et al.* 1982) and cells may increase their synthesis of cellular concentration with

increased UV exposure (Dunlap *et al.* 1986). MAAs are also recognized as possessing antioxidant properties, thereby reducing the impact of reactive oxygen species (Foyer *et al.* 1994). High concentrations of MAAs have been found in selected dinoflagellates, prymnesiophytes, cryptomonads, Antarctic diatoms and raphidophytes (Davidson *et al.* 1996, Jeffrey *et al.* 1999, Klisch and Hader 2000, Neale *et al.* 1998), and they are most prevalent amongst surface bloom-forming species (Jeffrey *et al.* 1999).

The raphidophyte microalga *Chattonella marina* (Subrahmanyan) Hara et Chihara is known to cause fish mortality in Japanese waters (Okaichi 1989) and has recently been implicated in mortality of farmed finfish in South Australia (Hallegraeff *et al.* 1998). Marshall & Hallegraeff (1999) reported that isolates of *C. marina* from South Australia (UTCMPL01) and Japan (NIES-118) differ in their tolerance to high light. The Japanese isolate originating from turbid eutrophic waters had lower tolerance of high intensities of visible light(500 μmol $m^{-2}s^{-1}$) than the isolate from clear waters off South Australia. Here we examine differences in tolerance to PAR and UV irradiance by the same Japanese and Australian strains of *C. marina*.

6.3 Materials and methods

6.3.1 Algal cultures

A non-axenic clonal culture of *C. marina* (UTCMPL01) was isolated from Port Lincoln, South Australia, during a bloom in April 1996. A Japanese strain from the Seto Inland Sea in 1983 (N-118) was obtained from the National Institute of Environmental Studies (NIES), Japan. Additional cultures were sourced as follows; *C.. marina,* CMAU2, isolated from Port Lincoln, South Australia, 2001; CMNZ, Cawthron Institute (CAWR18), New Zealand, isolated form Wellington Harbor, 1999; *C. antiqua* CAJP, from the National Institute of Environmental Studies (NIES-1), Japan, isolated form the Seto Inland Sea, 1978; *C. subsalsa* CSMX, from the Provosali-Guillard Center for Culture of Marine Phytoplankton

(CCMP217), USA, isolated from the Gulf of Mexico, 1983. The cultures were maintained in GSe media (Blackburn *et al.* 1989) at 28 psu salinity, 150 µmol $m^{-2}s^{-1}$ light and 20 °C temperature. Both seawater and nutrients were filter sterilized into autoclaved culture vessels. A single parental culture in exponential growth phase of each strain was used as inoculum for each experiment. Cell concentrations were determined by counting a minimum of 200 cells per sample on a Sedgwick Rafter cell using a Zeiss Axiovert microscope at 100x magnification.

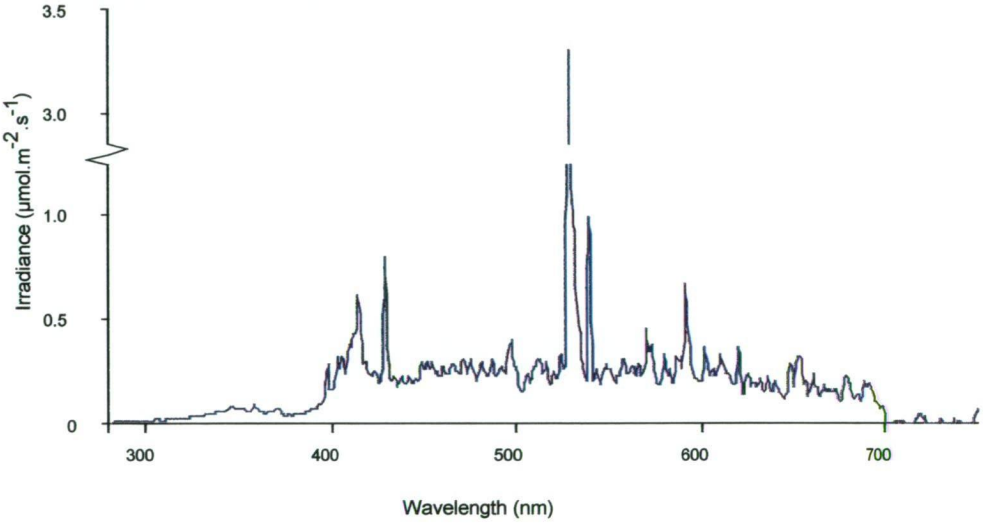

Fig. 1

Figure 6.1: Emission spectra (280-750 nm) of light sources used in experiments incorporating UVA, UVB and PAR at an irradiance greater than 300 µmol $m^{-2}s^{-1}$.

6.3.2 High PAR

High light intensity of 500 µmol $m^{-2}s^{-1}$ was provided using a metal halide lamp (DA 1500 A, 1500 V, 600W) on a 12:12 light:dark cycle at 25°C. Triplicate experimental cultures were grown in 5 l Ehrlenmeyer flasks containing 3 l of gently aerated GSe media. Controls were grown under the same conditions but using 150 µmol $m^{-2}s^{-1}$ cool white fluorescent light (Sylvania cool white). Light intensity was measured using a spherical 4π collector quantum light meter (Biospherical Instruments, QSL-100). The cultures were mixed and a 25 ml aliquot was withdrawn daily. One ml was transferred to a Sedgwick Rafter cell

for cell counts and a known volume (around 10 ml) gently filtered onto Whatman GF/F filter paper, stored at -70°C for determination of absorbance and pigment measurement. Experiments using other strains were grown under the above conditions in 125 ml Ehrlenmeyer flasks containing 75 ml of media.

6.3.3 UV treatments

Radiation of spectral properties similar to solar radiation was provided using 1 x Phillips 20W UVB, 3 x NEC 20W Blacklight UVA tubes and 6 x mercury halide lamps at 25 °C to produce an irradiance > 300 μmol $m^{-2}s^{-1}$ (Fig 6.1). Cultures were taken from maintenance conditions (day 0), and grown in 250 ml polystyrene disposable culture flasks (Falcon) containing 225 ml of GSe media.

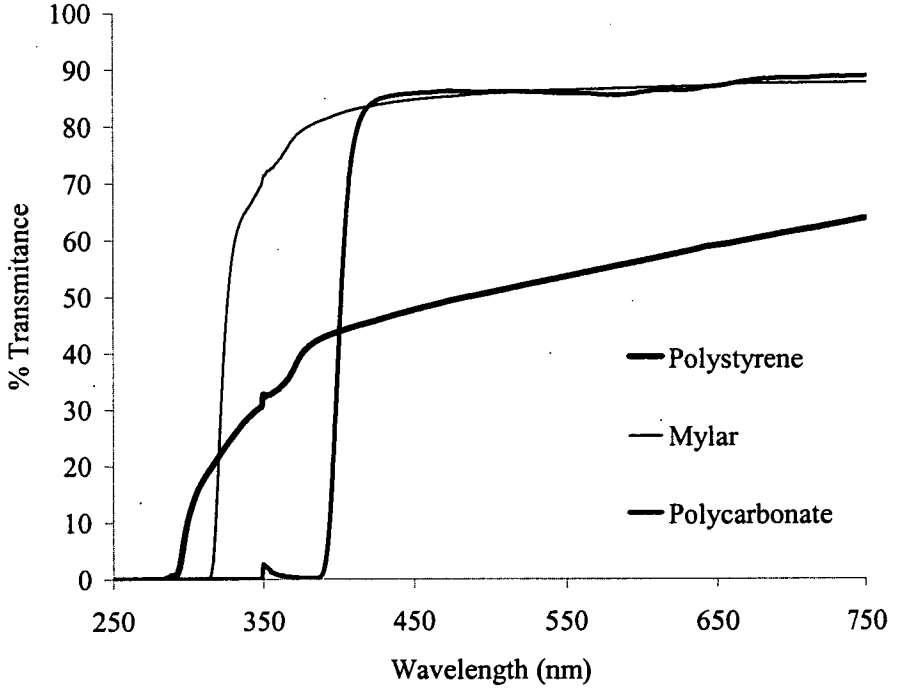

Figure 6.2: Spectral transmittance of UV absorbance screens between 250 and 750 nm.

Three UV treatments were obtained by removing ultraviolet wavelengths using a polycarbonate shield for PAR treatment (T > 400 nm), mylar shield for UVA + PAR treatment (0.007 mm; T > 320 nm) and all cultures grow in UVC screening polystyrene (Falcon; Transmission (T) > 295 nm) as shown in Fig. 6.2. Semi-continuous culture methods were used, with 25 ml aliquot replaced every 2-3 days with GSe media for 7 days. Samples were processed as described above.

6.3.4 Photosynthetic pigments and MAAs

Pigmented cells on filters were finely ground in 2 ml of HPLC grade methanol (BDH-Hipersolv) and filtered to 0.45µm (MFS syringe tip glass fibre filter). One ml of extract was used for spectrophotometric analysis (GBC UV vis spectrophotometer) at 250 to 750 nm, while the remainder was used for HPLC analysis. Chloroplast pigments were analyzed using HPLC according to Wright *et al.* (1991). The violaxanthin to zeaxanthin ratio used zeaxanthin readings multiplied by 1000. Analysis of MAAs used the following chromatographic conditions. Methanol extracts of 3 µl were injected into a Shimadzu HPLC system using a Phenosphere C-8 reverse phase HPLC column (Spheri-5, 4.6 mm i.d. x 25 cm) and a guard column of the same packing material (Spheri-5, 4.6 mm i.d. x 5 cm). The mobile phase was 40% methanol, 59.9% water and 0.1% acetic acid with a flow rate of 0.8 ml min-1 (Dunlap & Chalker 1986, Karentz et al. 1991). The detection wavelengths were 313 and 340nm. MAAs were identified by co-chromatography and comparison with known standards, provided by Dr W Dunlap of the Australian Institute of Marine Sciences.

6.3.5 Biological weighting

Light irradiance was measured each 1 nm between 280 and 800 nm using a Macam portable double grating spectroradiometer and weighted in accordance with the erythemal weighting function (McKinley and Diffey, 1987), because of its similarity to plant and DNA action spectra. As whole cells were used, a more specific physiological weighting function for the interactive response of the algal cultures was inappropriate. The erythemal action spectrum was also used to

weight the spectral and MAA absorbance data according to wavelength-specific biological effects from samples taken under UV treatment on days 0, 3, 5, and 7.

6.3.6 Statistics

Culture growth rate (k) was calculated as per Marshall and Hallegraeff (1999). Chl *a* absorbance at 665 nm was corrected for blank and background absorbance at 750 nm and Chl *a* concentration calculated using extinction coefficients from Porra et al (1997). The maximum MAA absorbance was normalized against Chl *a* giving a UV_{max}:Chl *a* ratio:

$$UV_{max}\text{:Chl } a = (\text{Abs. } UV_{max} - \text{Abs. 750nm}) / (\text{Abs. 665nm} - \text{Abs. 750nm})$$

Where UV_{max}:Chl *a* equals the ratio of blank corrected maximum spectrophotometric absorbance of MAAs between 280 and 390 nm divided by the blank corrected spectrophotometric absorbance of Chl *a* at 665 nm. Pigment ratios were compared using a Ryan-Einot-Gabriel-Welsch multiple range test for variability.

6.4 Results

6.4.1 High PAR

At high irradiance of 500 μmol $m^{-2}s^{-1}$ photoinhibition caused a decrease of *Chattonella* growth rates from 1.4 ± 0.9 to 1.0 ± 0.3 for the Australian and 0.9 ± 0.2 to 0.5 ± 0.04 for the Japanese strains, respectively. Spectrophotometric analysis showed a significant increase ($P\{F<0.05\}$) in the UV_{max}:Chl *a* of the Australian strain due to synthesis of MAAs under high light (Fig. 6.3). However, the UV_{max}:Chl *a* for the Japanese strain exposed to high PAR irradiance did not differ significantly from either the Japanese or Australian cultures grown under low light. Spectrophotometric absorbance of MAA production of other

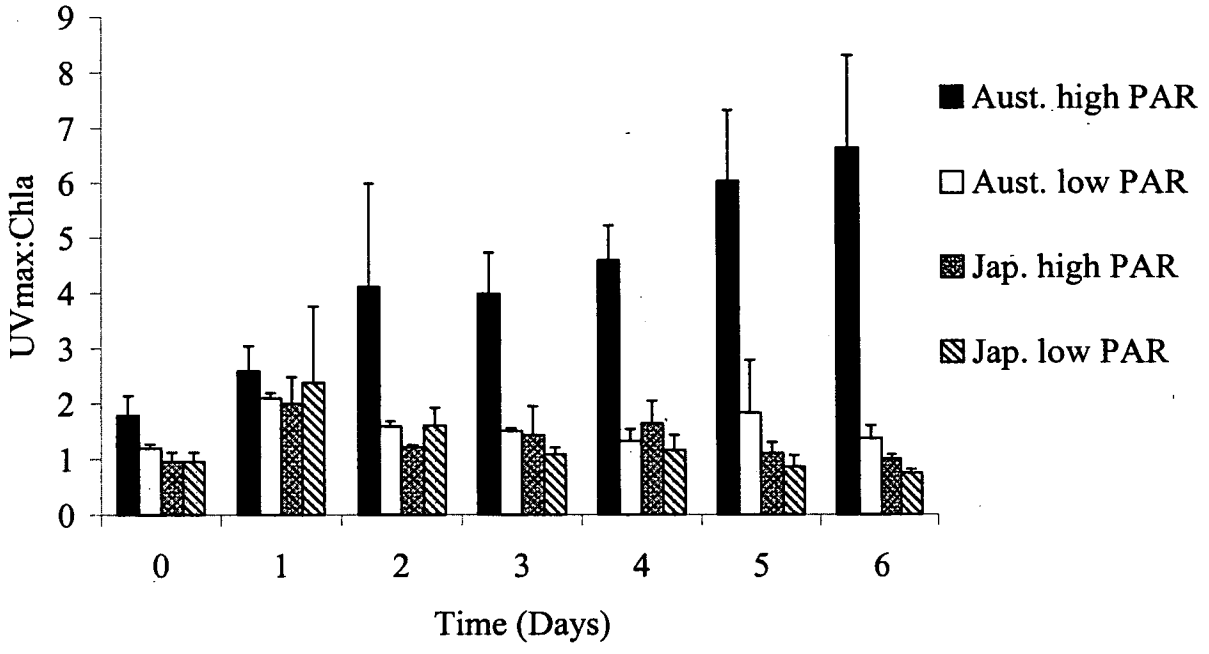

Figure 6.3: Ratios of absorbance by Chl *a* to MAAs (334:665 nm) for the Australian and Japanese strains of *C. marina* at high (> 500 µmol $m^{-2}s^{-1}$) and low (< 150 µmol $m^{-2}s^{-1}$) PAR irradiances.

Chattonella strains from Japan, New Zealand and USA indicated the Australian strains of *C. marina* were not unique in increasing sysnthesis of MAAs under high light intensity (Fig. 6.4). A recently (2001) isolated strain of *C. marina* from Port Lincoln (CMPL2) showed a similar response in MAA production to high irradiance as the original strain (CMPL). The New Zealand strain also showed an increase in MAA production under high irradiance (UV_{max}:Chl *a* from 0.76 to 0.79), whereas other Japanese and USA strains showed a decrease in MAA production.

Chlorophyll *a* production was higher in the Australian strain (9.3 ± 0.5 fg $cell^{-1}$ day^{-1}) than the Japanese strain (1.5±1.4 fg $cell^{-1}$ day^{-1}) under low light conditions. The Chl *a* per cell declined for both the Australian and Japanese strain exposed to

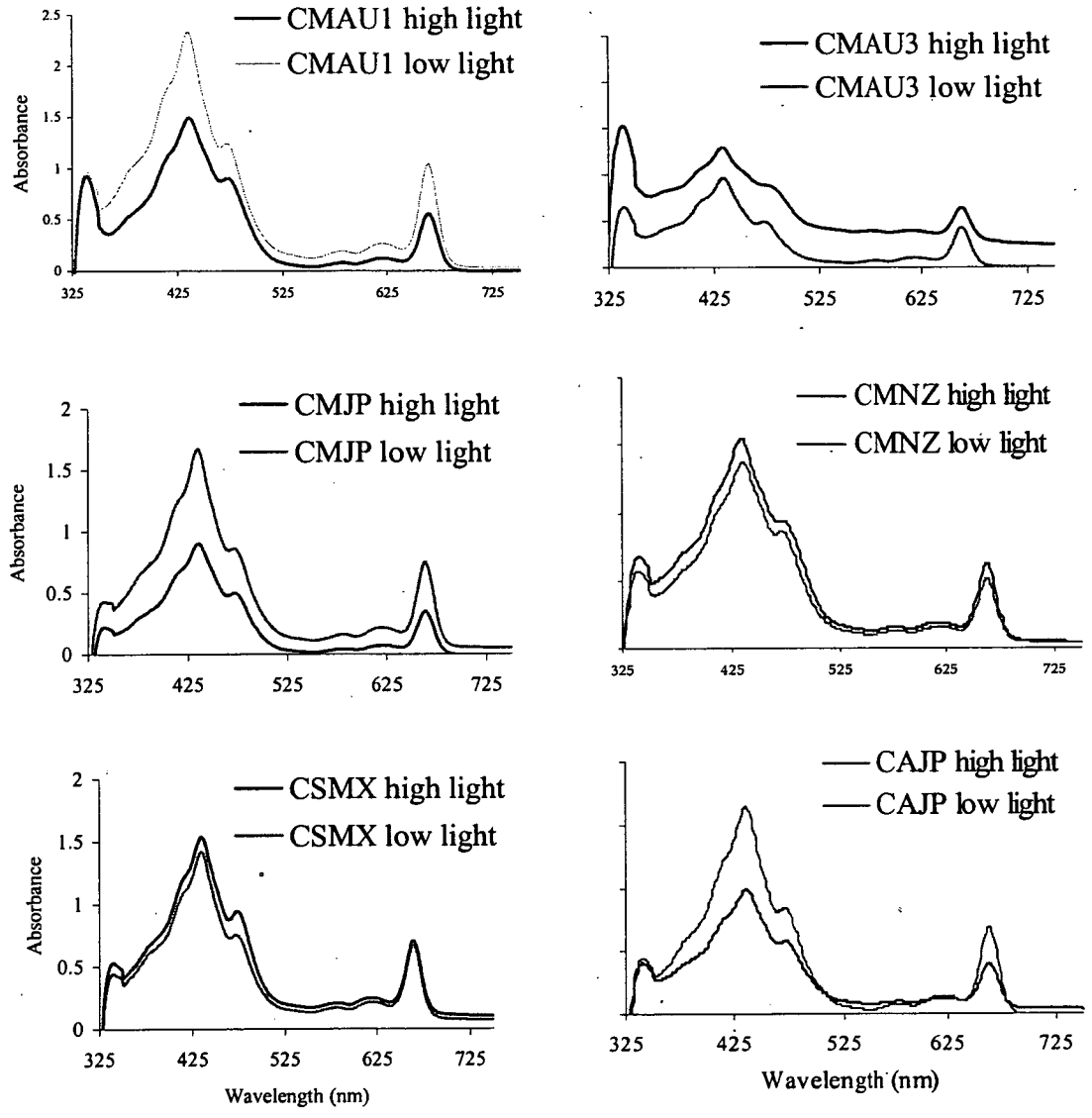

Figure 6.4: Pigment ratios of MAAs to Chl*a* (R334:665) as determined spectrophotometrically for 5 strains of *Chattonella spp.* under high (>500 µmol $m^{-2}s^{-1}$) and low (<150 µmol $m^{-2}s^{-1}$) irradiance. The Australian strain of *C. marina* (CMAU) shows the high R334:665, with the New Zealand (CMNZ) strain also showing increased MAA production under high irradiance conditions. Strains *of C. marina* from Japan (CMJP) and *C. subsalsa* from the Gulf of Mexico (CSMX) shoed a decrease in R334:665 when exposed to high light. The Japanese strain of *C. antiqua* (CAJP) was unable to survive the high irradiance conditions (n=5).

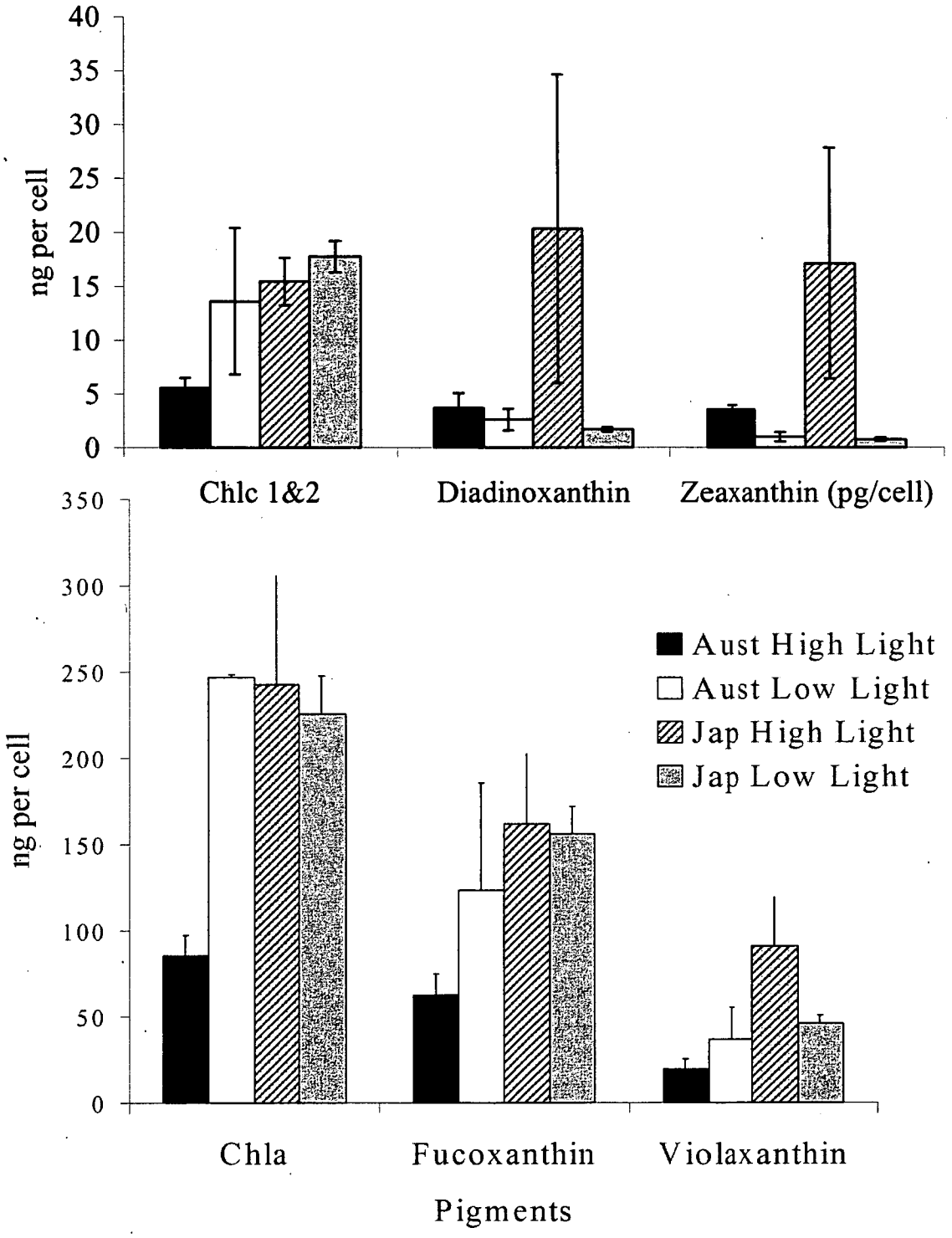

Figure 6.5: Pigment per cell as determined by HPLC for the Australian and Japanese strains of *C. marina* after 6 days exposure at high (> 500 µmol $m^{-2}s^{-1}$) and low (< 150 µmol $m^{-2}s^{-1}$) irradiances (mean ± SD, n=3).

high light. HPLC analysis showed that under high irradiance the Australian strain also responded with significant decreases in cellular concentrations of chlorophylls *c* 1 & 2 (from 13.61 to 5.55 ng $cell^{-1}$) and fucoxanthin (from 123.48 to 62.37 ng $cell^{-1}$) pigments (Fig. 6.5). The Japanese strain responded to high irradiance with increases in cellular concentrations of diadinoxanthin, zeaxanthin and violaxanthin (Table 6.3). No significant responses were noted in otherpigments to irradiance treatments.

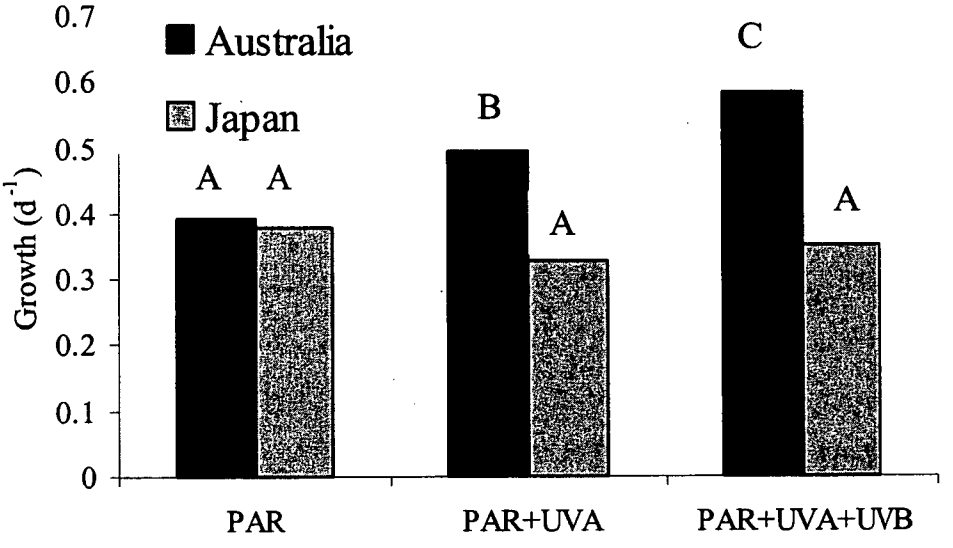

Figure 6.6: Growth rate of *C. marina* from Australia and Japan under different UV treatments (mean ± SD, n=3). Those values labled with the same letter are not significantly different at P<0.05.

6.4.2 UV Treatments

Growth rates of the Australian *C. marina* were highest in treatments exposed to PAR+UVA+UVB (0.59 ± 0.05 divisions per day, Fig. 6.6), declining to 0.50 ± 0.04 and 0.39 ± 0.03 divisions per day in the PAR+UVA and PAR treatments respectively. No difference in growth was observed between UV light treatments for the Japanese strains. There was a significant difference between growth rate of strains (P< 0.001) and a highly significant interaction between strain and treatment (P, 0.0006).

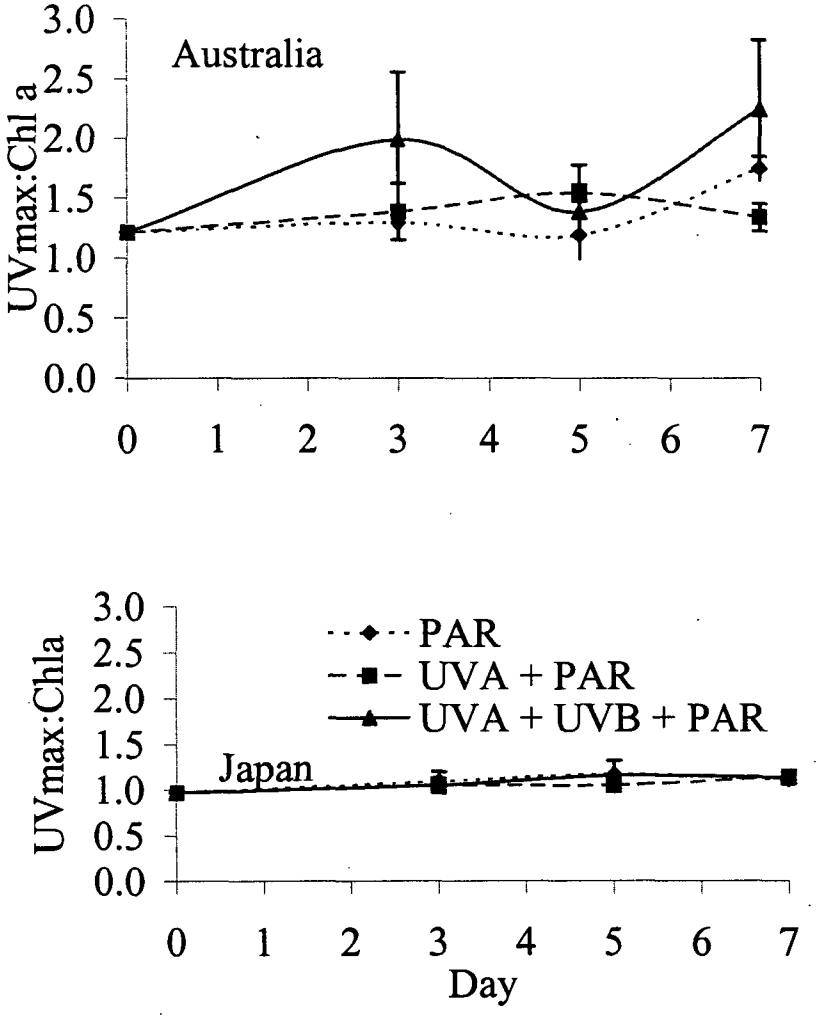

Figure 6.7: Pigment ratios of MAAs to Chl *a* (R334:665 nm) as determined spectrophotometrically for Australian and Japanese strains of *C. marina* under treatments of PAR (transmission (T) > 400 nm), PAR+UVA (T > 320 nm) and PAR+UVA+UVB (T > 295 nm), (mean ± SD, n=3).

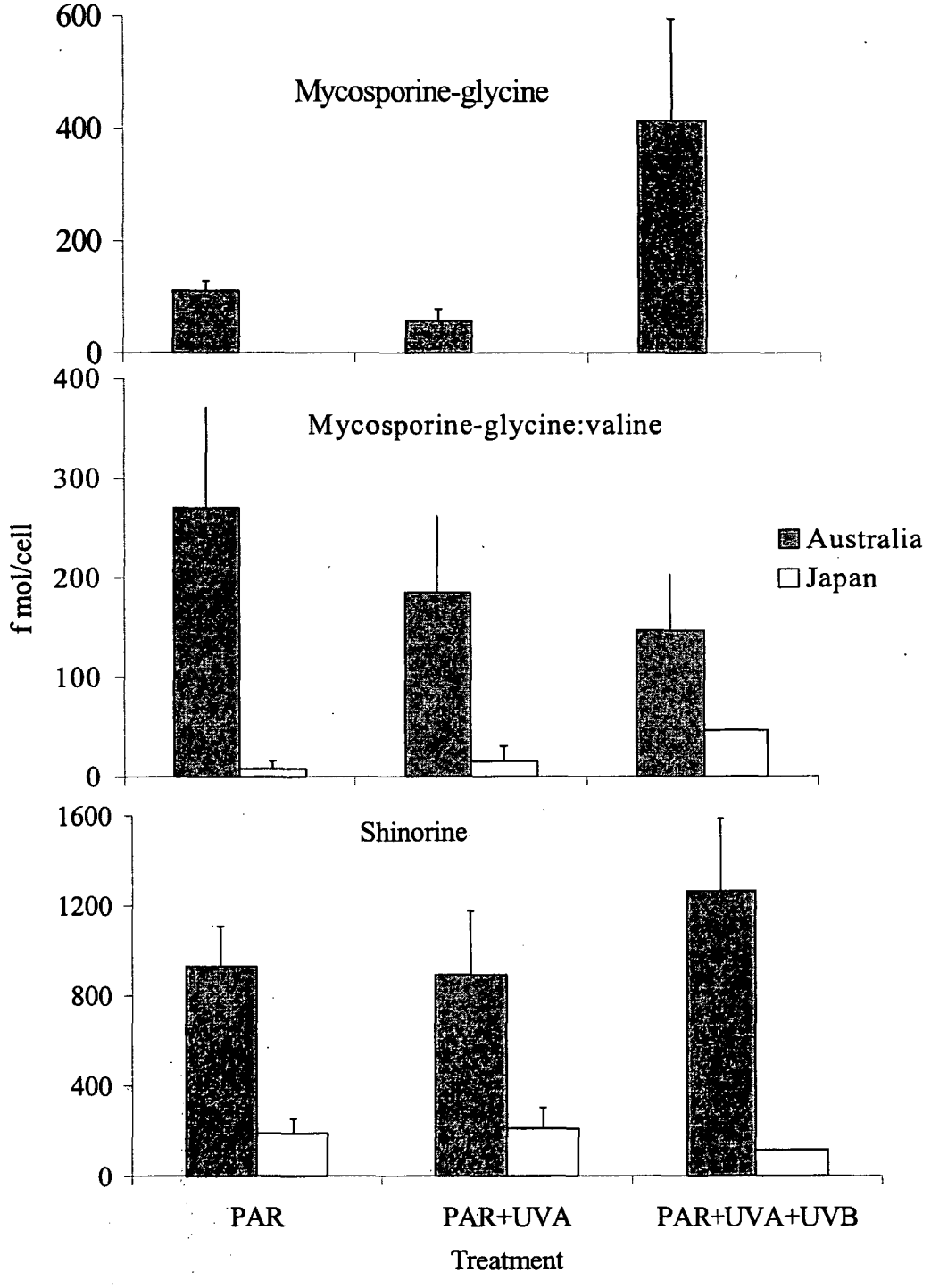

Figure 6.8: Concentrations of MAAs on day 3 of incubation for each treatment. a) mycosporine-glycine; b) mycosporine-glycine:valine; c) shinorine. Note different scales on y-axes.

The ratio of absorbance at UV_{max}:Chl *a* for the Australian strain increased with time under UV treatment (Fig. 6.7) when compared with UV screened treatments. Japanese strains showed no significant increase in the UV_{max}:Chl *a* ratio irrespective of light treatment. The level of Chl *a* production for each strain showed no significant difference between treatments, however the Chl *a* production per day for the Australian strain was 4 to 18 times higher than the Japanese strain under corresponding treatment.

HPLC results showed the presence of the MAAs mycosporine-glycine, shinorine and mycosporine-glycine:valine in the Australian strain, with the Japanese strain having an order of magnitude less shinorine and mycosporine-glycine:valine and no detectable mycosporine-glycine (Fig. 6.8). Concentrations of mycosporine-glycine per cell were significantly higher in the treatment exposed to PAR+UVA+UVB than the treatment where UVB was excluded for the Australian strain UVB exposure reduced mycosporine-glycine:valine concentrations in the Australian strain but enhanced their concentrations of this compound in the Japanese strain. . Shinorine levels were also highest in treatments exposed to UVB wavelengths.

6.4.3 Biological weighting function

The erythemal UV irradiances (Table 6.1) approximate a water column depth of 0-15 meters in clear oceanic waters. Exposure of the Australian strain to UVB caused a doubling in the erythemally weighted MAA absorbance (Table 6.2), but treatments excluding UVB caused a significant reduction in the erythemally weighted MAA absorbance (72.49 per 10^6 cells to 0.07 per 10^6 cells). The Japanese strain showed no significant variation in erythemally weighted MAA absorbance irrespective of light treatment, remaining at levels three orders of magnitude lower that the Australian PAR+UVA+UVB treatment.

Table 6.1. Erythemal UV-attenuation screens and equivalent water column depth (in meters) experienced by algal cultures for each experimental light treatment calculated using Beer's Law ($I/I_o=e^{-kz}$). I_o and I are the erythemal irradiance at the surface and z meters depth respectively and k for erythemal UV was 0.4 (mean for low turbidity oceanic waters).

Irradiance Screen	Wavelength	Erythemal UV ($W.\ m^{-2}$)	Equivalent South Australian water column depth (m)	Equivalent Japanese water column depth (m)
Sunlight Aust[1]	PAR + UVR	3.085×10^{-2}	0	-
Sunlight Japan[2]	PAR + UVR	2.900×10^{-2}	-	0
Polystyrene	PAR+UVA+UVB	1.91×10^{-2}	1.199	1.044
Mylar	PAR+UVA	2.95×10^{-3}	5.868	5.714
Polycarbonate	PAR	7.74×10^{-4}	9.213	9.059

[1]Gies *et al.* 1994; [2]Ono 1997

6.5 Discussion

6.5.1 Photoprotection by Australian and Japanese strains

Marshall & Hallegraeff (1999) found that, even after gradual conditioning, the Japanese strain did not have the ability to grow at irradiances above 200 µmol $m^{-2}s^{-1}$ while the South Australian strain did not grow under irradiances less than 50 µmol $m^{-2}s^{-1}$. Similarly, Khan (1998) found that other Japanese strains from Kagoshima Bay, Japan, also lacked the ability to tolerate high irradiances. In the present work we show that a Japanese strain of *C. marina* contained low concentrations of MAAs irrespective of light treatment while exposure of the Australian strain to UVB caused biologically weighted UV absorbance by MAAs to increase over 3 orders of magnitude. The occurrance of MAAs correlated with increased UV tolerance, growth rate and Chl *a* per cell. MAAs absorb ultraviolet wavelengths, including UVB (Helbling 1996, Davidson 1998), and may also play an antioxidant role (Dunlap and Yamamoto, 1995). The Japanese strain of *C.*

marina does not contain high concentrations of MAAs and exhibits no capacity to synthesise more under UV-exposure. We found that this strain was more vulnerable to UV-induced cell damage, inhibition of photosynthesis and growth, and which may lead to cell death. The presence of high levels of MAAs in the Australian strain of *C. marina* suggests that it is adapted to bloom under conditions of high UV in near-surface waters. Jeffrey *et al.* (1999) reported significant strain differences in UV_{max}:Chl *a* absorbance for the toxic bloom forming dinoflagellate *Gymnodinium catenatum* (Japan 1.83; Tasmania 2.17; Portugal 6.75). Such differences are thought to be due to the local light environment selecting for different ecophenotypes. Similary, in the present work, differences in the photophysiology of isolates of *C. marina* from South Australian and Japanese waters are likely to correlate with the characteristics of the light environment from which they were isolated. Erythemal UV irradiances at the Earth's surface in Japan and South Australia are similar, being around 2.900 and 3.085×10^{-2} W. m^{-2} respectively (Ono 1997, Gies *et al.* 1994). However, the Japanese strain was isolated from the eutrophic Seto Inland Sea where the turbid waters limit penetration of solar radiation. The minor increase of UV_{max}:Chl *a* in the New Zealand strain of *C. marina* may be due to the strain originating from the metropolitan environment of Wellington Harbor, which may have less solar radiation and greater water turbidity than Port Lincoln, South Australia. These differences suggest that local environmental conditions influence the light climate and determine the photophysiology of these strains.

Biologically weighted light absorption by cells suggests little or no photo-induced synthesis of MAA by the Australian strain under treatments without UVB. Furthermore, it indicates substantial degradation of MAAs to a greatly reduced cell specific absorption after 3 days incubation. The concentration of MAA is a dynamic equilibrium between synthesis and degradation. The increase in MAAs observed in the PAR + UVA + UVB occurred in addition to the MAA synthesis required to maintain such equilibrium concentration. These results indicate the decline in MAA concentration was independent of light conditions and may be

due to bacterial degradation (Marchant *et al.* 1991). This indicates that UVB increases the synthesis of MAAs. Unlike many plankton, the Australian strains of *C. marina* responded to UVB irradiance and any increase in these wavelengths due to ozone depletion will elicit an appropriate increase in protection.

6.5.2 Xanthophyll cycle

Although unable to significantly mitigate UV-induced damage using MAAs, the Japanese strain responded to high irradiance (500 μmol $m^{-2}s^{-1}$) by increased cellular levels of the carotenoid zeaxanthin, which suggests the presence of a xanthophyll cycle in this strain. The three-component xanthophyll cycle, previously reported only for higher plants, green and brown algae, allows photosynthesis to be maintained by quenching the excited singlet state of chlorophyll at high PAR fluxes (Porra *et al.* 1997). The Australian strain contained only 21% of the violaxanthin and zeaxanthin found in the Japanese strain under high light intensities, but the ratio of these pigments did not differ significantly between high and low light irradiances (Table 6.3). Concentrations of violaxanthin and zeaxanthin found in Australian and Japanese isolates grown at low irradiance were comparable with those found in two Japanese isolates (N-14 and N-121) grown at 75 μmol $m^{-2}s^{-1}$ by Mostaert *et al.* (1998)

6.5.3 Growth enhancement of the Australian Chattonella by UVB

The present work demonstrates that UVB radiation enhanced growth by 150% compared to PAR for the Australian isolate. The colonial stage of the Antarctic prymnesiophyte *Phaeocystis antarctica* Karsten, which contains high concentrations of MAAs, has also been found to increase its growth rate in the presence of UV, potentially giving it a competitive advantage in surface blooms (Davidson *et al.* 1996). UV-absorbing compounds have also been found in dinoflagellate species that dominate surface blooms such as *Gymnodinium catenatum* (Jeffrey *et al.* 1999) and *G. sanguineum* (Neale *et al.*, 1998).

Table 6.2; Erythemally weighted absorption by UV absorbing compounds per 10^6 cells for the Australian and Japanese strains of *C. marina* (mean ± SD, n=3).

Day	PAR		PAR+UVA		PAR+UVA+UVB	
	AUST	JAPAN	AUST	JAPAN	AUST	JAPAN
0	72.49 ± 0.00	0.06 ± 0.00	72.49 ± 0.00	0.06 ± 0.00	**73 ± 0**	0.06 ± 0.00
3	0.09 ±0.02	0.06 ± 0.01	0.11 ± 0.01	0.06 ± 0.02	**139 ± 68**	0.07 ± 0.01
5	0.15 ± 0.02	0.03 ± 0.00	0.10 ± 0.02	0.03 ± 0.01	**95 ± 26**	0.06 ± 0.01
7	0.13 ± 0.05	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.02	**148 ± 73**	0.07 ± 0.02

The coincidence of MAAs in species that form near-surface blooms indicates that they may contribute to their dominance in near-surface waters.

The Japanese *Chattonella* strain showed no enhancement of growth under treatments containing UV radiation (Fig. 6.5) in spite of possessing low levels of UV screening MAAs. The UV radiation level of the treatments was equivalent to a water column depth of 0.167m (Table 6.1), which would naturally be encountered by this bloom forming species. This species is well adapted for near-surface blooms in a turbid, low UV environment. The erythemal weighting of the absorption spectra of the MAAs (Table 6.2) clearly demonstrates the ability of the Australian isolate to tolerate higher UV radiation, than the Japanese isolate.

Table 6.3; Cellular concentration and ratio between the carotenoid pigments violaxanthin and zeaxanthin (R Viol:Zea x 1000) in Australian and Japanese isolates of *Chattonella marina* treated with high and low irradiances. n=3.

	Australian (ng/cell)		Japanese (ng/cell)		Australian R viol:Zea	Japanese R Viol: Zea
Treatment	Viol.	Zea.	Viol.	Zea.	(x 1000)	(x 1000)
High Irradiance	19.29	3.56	90.70	17.14	7.05	8.26
(>500 µmol $m^{-2}s^{-1}$)	± 6.03	± 0.41	± 28.30	± 10.73	± 1.18	± 3.36
Low Irradiance	36.60	0.99	45.81	0.76	67.01	63.91
(<150 µmol $m^{-2}s^{-1}$)	± 18.38	± 0.44	± 4.65	± 0.16	± 26.55	± 9.27

Chattonella marina is a subsurface bloom forming, highly motile flagellate, capable of active vertical migration. The decreased production of MAAs in the Japanese isolate of *C. marina* suggests that this strain may need to vertically migrate in the turbid waters of the Seto Inland Sea to avoid UV exposure. However, the Australian strain, inhabiting waters that are penetrated by UV wavelengths to greater depths, may have developed alternative UV-tolerance strategies. Tolerance of high PAR and UV appears to allow the Australian strain, known to prosper in near surface waters, potentially outcompeting co-occurring phytoplankton species that are less UV tolerant. Ozone depletion and the consequent increasing UV radiation in the Southern Hemisphere thus potentially could increase the concentration of *C. marina* relative to less UV-tolerant phytoplankton species, increasing the threat to the finfish aquaculture industry .

6.5.4 MAAs, Reactive Oxygen Species and implications for aquaculture

A range of antioxidants that been identified that protect cells against UV-induced reactive oxygen species (ROS) (Foyer et al. 1994). The production of mycosporine-glycine has been linked to antioxidant activity in invertebrate-algal symbionts of corals (Dunlap and Yamamoto 1995). Mycosporine-glycine may be capable of reacting with alkylperoxyl radicals to inhibit radical-chain lipid peroxidation and may provide some protection against photo-oxidative stress

induced by oxygen radicals in photoautotrophic symbiosis (Dunlap & Yamamoto 1995). The raphidophyte *C. marina* is well known for its toxicity to fish. Production of reactive oxygen species (ROS) is thought to contribute to its toxicity (Marshall *et al.* 2001, Oda *et. al.*, 1998). Therefore the increase in MAAs may moderate production of ROS, decreasing ichthyotoxicty of this species. Potential links between high irradiance, the production of ROS, antioxidant MAAs and *C. marina* toxicity have previously been suggested (Marshall *et al.* 2001). Production of ROS by UVR and the role of MAAs in quenching these ROS is worthy of further examination.

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Chapter 7

Effect of irradiance on superoxide production by *Chattonella marina* (Raphidophyceae) from South Australia and Japan*

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7.1 Abstract

Previous autecological studies demonstrated that Japanese and Australian strains of the raphidophyte *Chattonella marina* had similar tolerances for temperature and salinity, but substantial differences in irradiance requirements [1]. In the present work, differeing superoxide production and toxic effects on the zooplankton *Artemia salina*, and fish *Onchorynchus mykiss* are documented between geographic strains and light treatments. The fish killing mechanism of *C. marina* is suggested to be a synergistic effect between reactive oxygen species (ROS) and neurotoxins, and the results cannot be explained on the basis of these mechanisms of toxicity on their own.

7.2 Introduction

Fish mortalities caused by raphidophycean flagellates are a serious problem for aquaculture in the United States, Canada, New Zealand and Japan [2]. The first Australian report of a *Chattonella* associated fish mortality was in April 1996 in Boston Bay, South Australia [3] when approximately 1,700 tonnes of caged southern bluefin tuna (*Thunnus maccoyii*) worth $A45 M died coincident with a *Chattonella marina* bloom of 6.6 x 10^5 cells per litre. Blooms of *Heterosigma* and *Chattonella* have remained a problem to fin-fish aquaculture worldwide despite being widely researched in Japan for the last 2 decades [2], however, the killing mechanism of *Chattonella* blooms remains unclear. Initial theories for toxicity centered around anoxia, mucus production, respiratory, ionoregulatory and cardiovascular physiology of fish exposed to *Chattonella* blooms [4,5,6]. Histopathology showed severe changes in fish gill pathology when exposed to *Chattonella* [7]. Studies have also centered on the production of a toxin similar to breve toxins produced by *Gymnodinium breve* [8,9]. More recent studies have shown that some raphidophytes produce reactive oxygen species (ROS) such as superoxide ($\cdot O2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical $\cdot OH$ [10,11] implicated in fish mortality.

In this study we investigate the role that irradiance may have on superoxide production by *C. marina*, and determine the differences between the Australian and Japanese strains

in ROS toxicity to marine organisms. The relationship between ROS and neurotoxin production in fish mortalities is also discussed.

7.3 Methods

7.3.1 *Algal Culturing*

Stock cultures of *Chattonella marina* from Japan (NEIS-118) and Australia (CMPL01) were isolated and maintained as per Marshall & Hallegraeff [1]. Treatment cultures were grown as follow; zooplankton bioassays - 125 ml conical flasks under cool white fluorescent tubes (150 µmol m-2s-1) or metal halide lamps (500 µmol m-2s-1); fish bioassay - aerated 3L glass or 20 L polycarbonate vessels under light conditions as above. Unless stated, experiments were conducted using early to mid exponential phase cultures.

7.3.2 Animal Bioassays

Four day old *Artemia salina* meta-nauplii were introduced to 3 ml of *C. marina* culture. Treatments of high and low irradiance as described above, and controls of GSe media and culture with 500 units/ml catalase (Cat) and 50 units/ml superoxide dismutase (SOD) were in triplicate. Observations of toxicity were assessed as death, response, and no response. Response was defined as paralysis or reduced respiratory movements. Rainbow trout smolts (*Oncorhynchus mykiss*: 18-61 grams) were conditioned to 25 psu salinity over a period of 7 d. Fish were exposed to Australian and Japanese *C. marina*, at cell densities of 5-7 x 10^6 cells L^{-1}, under high (> 200 µmol $m^{-2}s^{-1}$), low (< 50 µmol $m^{-2}s^{-1}$) irradiances, and dark, with 1 hr preconditioning treatments (ethics approval 98081 - University of Tasmania). Australian *Chattonella* cell free extract was prepared through gravity filtration. Observations were taken until morbidity or death occurred. Surviving fish were sacrificed at 6 hrs. Gills were removed and processed using routine methods, stained with H&E and Alcian blue-periodic acid Schiff (PAS). Gill tissue was analysed by light microscopy to determine extent of lesions, epithelial separation and mucus cell disruption.

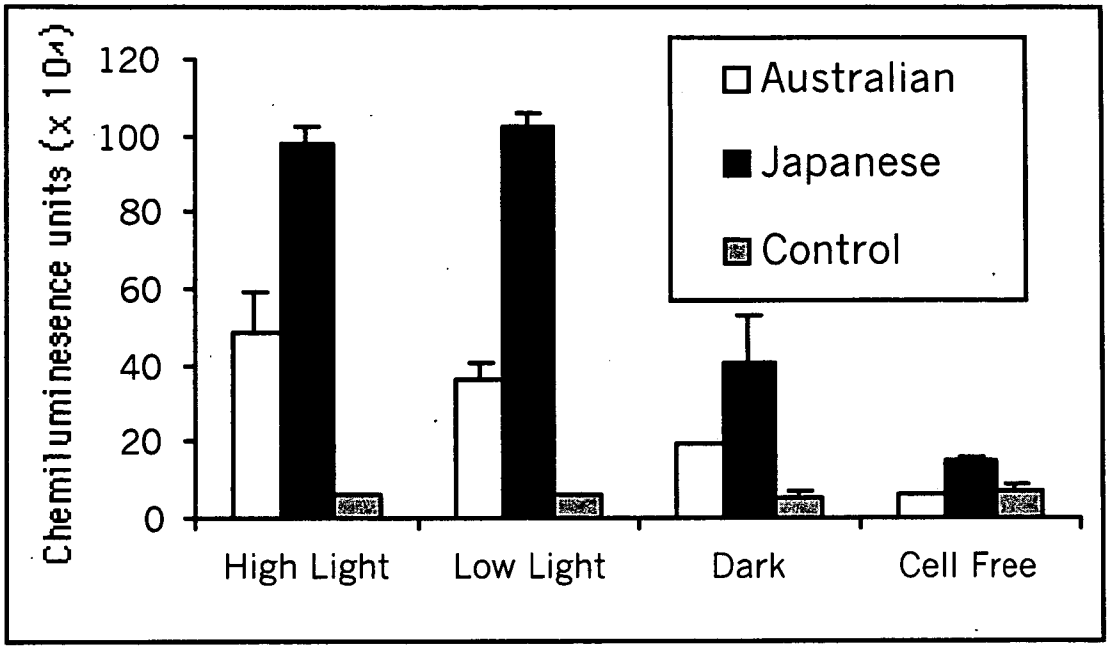

Fig. 7.1. Superoxide production of *C. marina* in response to different irradiances

7.3.3 Chemiluminescence Analysis

The oxygen radical superoxide (O2-) was measured using the luciferin analogue 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) as described by Lee et al. [12] at a concentration of 5 x 10-6M and standardised against 5 x 10-6 M superoxide dismutase (SOD) . Each measurement was made in triplicate.

7.4 Results

7.4.1 ROS Production

The Japanese strain produced a higher level of superoxide under both high and low irradiances compared to the Australian strain, but with no significant difference between high and low irradiance treatments. There was a significant decrease between the high and low light exposures and the dark treatments in both the Japanese and Australian strains (Fig. 7.1). The Australian strain however exibits a significant decrease in superoxide production between the low irradiance treatment and the dark treatment, with

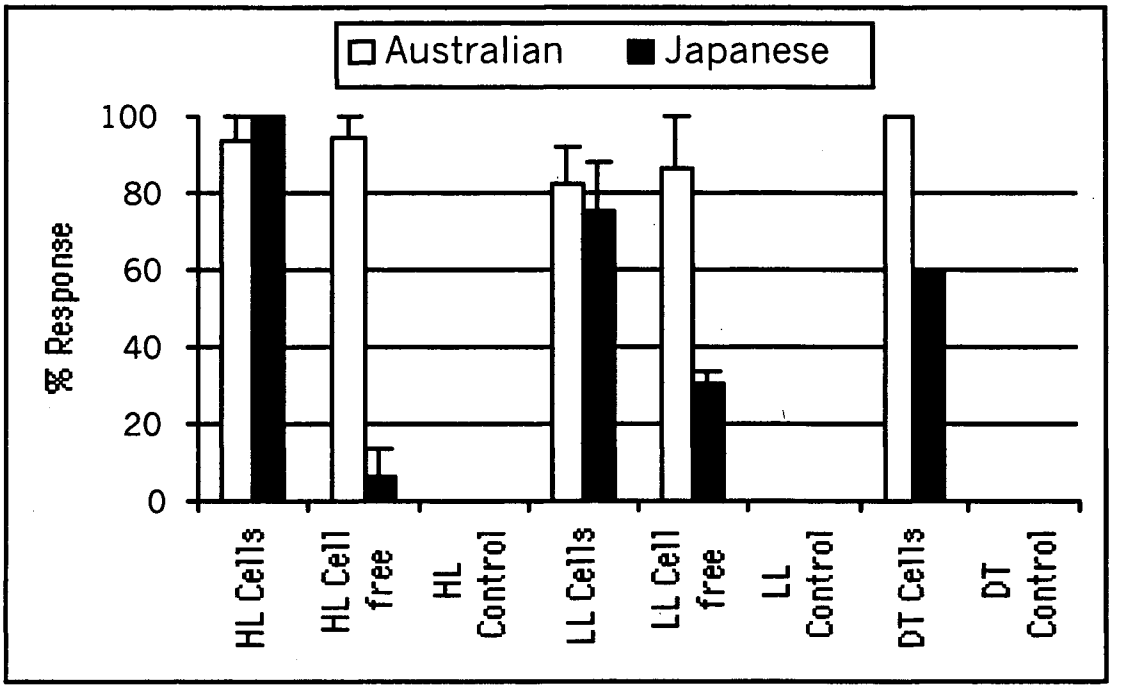

Fig 7.2: Toxic response of *Artemia salina* when exposed to *Chattonella marina.*

the dark treatment insignificant from the control. The cell free extracts of *C. marina* showed superoxide production of 6-8 times less than whole cell samples, relative to each treatment and strain. Cell free production of superoxide was significantly higher ($p>0.05$) than the controls.

7.4.2 Artemia Bioassay

Death did not occur in the *Artemia* exposed to *C. marina* within the experimental time frame. Meta-nauplii of *Artemia* showed a maximum toxic response (paralysis, clumping or mucus) to *C. marina* at culture age 8 to 27 d (exponential phase). No toxic response was noted for an aged (90 d stationary stage) culture or in controls (data not shown).

Response of *Artemia* after 6 hrs exposure to high or low light cultures was not significantly different between either Australian and Japanese strains (Fig. 7.2), and no decrease in toxic response was noted with the addition of the enzymes SOD and catalase

(data not shown). However *Artemia* showed a reduced response to the Japanese strain for the dark treatments and cell free treatments when compared to the Australian strain.

7.4.3 Fish Bioassays

Initial excitability, loss of balance, rapid opercular movements and a decrease in activity were symptoms exhibited within 15 minutes exposure to *C. marina* (Table 7.1). There was no significant difference in fish mortality between high and low irradiance treatments, with most mortalities occurring within 2 hrs. The rate of opercular movements was highest in fish exposed to Japanese strains under high irradiance, and greater for both Australian and Japanese strains with increased irradiance (Table 7.2). Dark treatment enhanced fish survival, with only 1 mortality out of 4 occurring. There was no mortality in the cell free extract. Fish mucus was noted in all treatments except dark treatment, cell free extract and controls. A twisting of the posterior spine, thought to be due a neurotoxic effect, was noted in all treatments including the cell free extracts, but absent in the controls. Histopathology revealed no significant damage in gill lamellae consistent with ROS. Although some effect was noted between the treatments and controls, there were no quantifiable histological changes.

Table 7.1; Response of rainbow trout (*Onchorynchus mykiss*) when exposed to the ichthyotoxic flagellate *Chattonella marina*..

Treatment		n	Time until fish responses (mins)				
			Opercular movements (beats/minute)	Loss of balance	Production of mucus +low +++high	Involuntary flexion	Death
High Light	Control	4	0	0	0	0	0
	Aust	4	15	15	15 +++	15	95 ± 9
	Jap	4	45	30	15 ++	0	127 ± 45
Low Light	Control	4	0	0	0	0	0
	Aust	4	15	15	15 +++	0	75 ± 30
	Jap	4	60	0	15 +++	15	105 ± 64
Dark	Aust	4	ND	ND	1 @15 +	15	1 @ 140
	Jap	4	ND	ND	0	0	0
Cell Free	Aust	4	0	0	0	15	0

Table 7.2: Opercular movements per minute in *Onchorynchus mykiss* exposed to *Chattonella marina* under high and low irradiances (n=8).

	Australian	Japanese	Control
High Irradiance (> 200 μmol $m^{-2}s^{-1}$)	132±11.3	152±0	no data
Low irradiance (< 50 μmol $m^{-2}s^{-1}$)	116	112	110±14.1

3.5 Discussion

3.5.1 Relationship between light intensity and ROS toxicity

Although the zooplankton *Artemia* showed no acute toxicity from *Chattonella* cells, paralysis of *Artemia* occurring in treatments with and without the enzymes SOD and Catalase indicate that 1) *Artemia* are not susceptible to ROS toxicity and 2) there is another toxic principle involved (most likely a neurotoxin) influencing *Artemia* behaviour, but not causing death. Medlyn [13] tested adult *Artemia* against 4 strains of *Gymnodinium breve* (Davis) extract which produces three toxic components, PbTx-2, PbTx-3 and PbTx9, lethal to *Artemia* after 72 hours exposure. These three toxic components are similar to those produced by *Chattonella marina* [9]. The raphidophyte *Heterosigma akashiwo* has also been found to have lethal toxicity to *Artemia* from both cultures and cell filtrates [14], and is known to produce relatively low levels of superoxide and hydrogen peroxide compared to *C. marina* [15]. The effect of the *C. marina* culture on *Artemia* is more likely to be of neurotoxic origin than from ROS'. *Artemia* displayed the same paralysis response in both Australian whole cell and cell free extract, indicating that the toxicity component is present outside the cells. The significantly lower response in *Artemia* from the Japanese cell free and dark treatments suggests that the Japanese strain produces much lower amounts of the neurotoxins.

There was a moderate toxic effect of both Japanese and Australian *C. marina* upon rainbow trout. The fish exhibited symptoms consistent with the production of ROS such as mucus production, but not at the intensity as described by [16], and limited gill pathology indicates that the levels of ROS production were not lethal. However, the presence of superoxide was confirmed through the chemiluminesence assay.

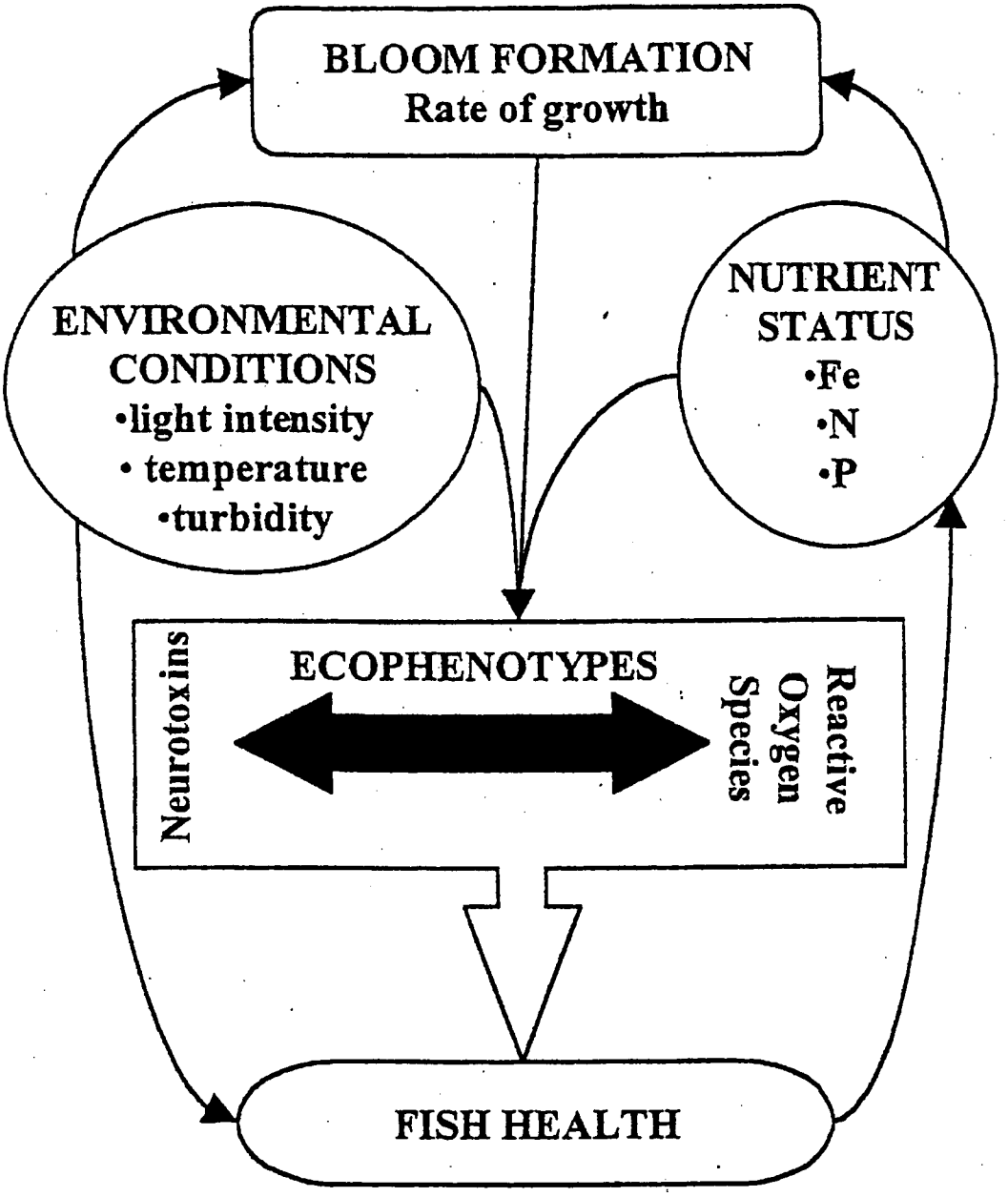

Fig. 7.3 Flow diagram demonstrating possible exogenous effects upon *C. marina* toxicity

Opercular movements did increase under both high and low light intensity, indicating that the fish were in respiratory distress. This observation was also reported for bream (*Pargus major*) exposed to *C. marina* [6]. The symptoms displayed by fish exposed to cell filtrate compare with those described by Ahmed and Onoue [17], were fish where exposed to a crude toxic extract from *C. marina.*

7.6 Conclusion

A synergistic effect of neurotoxins and reactive oxygen species toxicity by *C. marina* is suggested by the variation in toxic impact on different organisms under changing irradiances. There is also evidence that different strains (ecophenotypes) may simultaneously produce different toxic effects. Relationships between growth phase, temperature light, iron, and both ROS and neurotoxin production have previously been reported [8,9,18]. The development of a bloom prediction model may need to incorporate not only traditional physical factors such as stratification, water temperature, and eutrophication, but also irradiance, as demonstrated in Fig. 7.3.

In monitoring fish mortality events, involvement of both ROS and "breve-like" toxins needs to be studied concurrently rather than in isloation.

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Chapter 8

Photosynthesis Does Influence Superoxide Production in the Ichthyotoxic Alga *Chattonella marina* (Raphidophyceae)*

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8.1 Abstract

The ichthyotoxic flagellate *Chattonella marina* produces extraordinary levels of the reactive oxygen species (ROS) superoxide, which is partially controlled by electrons donated through photosynthetic electron transfer. We demonstrate ROS can be significantly reduced using the photosynthesis blocking herbicide DCMU. As superoxide is considered a contributor to the toxicity of C. marina, fish mortalities may be more prominent during daylight hours.

8.2 Results and Discussion

Raphidophyte algae are red tide phytoplankton frequently associated with finfish mortalities in Australia, Canada, Japan, New Zealand and the United States (Chang *et al.* 1990, Hallegraeff *et al.* 1998, Okaichi 1997, Taylor and Horner 1994). The precise mechanism of toxicity from these organisms remains unclear, but the production of reactive oxygen species (ROS) is suggested as one key factor contributing to fish mortalities (Ishimatsu *et al.* 1996, Marshall *et al.* 2001, Oda *et al.* 1997, Tanaka *et al.*, 1994, Yang *et al.* 1995). Reactive oxygen species such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) cause damage to gills through lifting of the gill epithelial layer and reducing respiratory exchange (Shimada *et al.* 1991).

Most raphidophyte species produce $\cdot O_2^-$ and H_2O_2 under non-stressed conditions without triggers or stimulants (Oda *et al.* 1998). The rate of ROS production in *Chattonella* spp. is the highest in the raphidophyte group (Table 8.1), with the comparatively large *C. marina* and *C. antiqua* cells producing an order of magnitude more $\cdot O_2^-$ and up to 25% more H_2O_2 than *Heterosigma akashiwo* (Oda *et al.* 1998). Maximum ROS generation occurs during the exponential stage in *C. marina* (Kawano *et al.* 1996), as well as in the dinoflagellate *Cochlodinium polykrikoides* (Kim *et al.* 1999a), suggesting that oxygen radicals are released during active cellular metabolic processes.

Table 8.1: Production rates of superoxide and hydrogen peroxide by raphidophyte and other flagellates elucidated from the literature

Species	Superoxide nmol / 10^4 cells / min	Hydrogen peroxide nmol / 10^4 cells / min
RAPHIDOPHYTE		
Chattonella antiqua	1.1[1]	0.12[1]
Chattonella marina	2.68[1]	0.7[1]
	1.95[5]	0.46[5]
Fibrocapsa japonica	< 0.1[1]	>0.1[1]
Heterosigma akashiwo	< 0.1[1]	0.80 ± 0.01[1]
		0.005[3]
	0.026[4]	0.020[4]
	0.018 ± 0.001[9]	0.027 ± 0.002[9]
		0.008[2]
Olisthodiscus luteus	< 0.1[1]	< 0.1[1]
	0.011 ± 0.002[9]	0.017 ± 0.006[9]
DINOFLAGELLATE		
Cochlodinium polykrikoides	0.55[6]	1.2[6]
HAPTOPHYTE		
Hymenomonas caterae	negligible[8]	0.017-0.033[8]
GREEN ALGA		
Tetraselmis apiculata	N.D.	0.00003[2]
Surface ocean water	N.D.	0.01-0.20 nmol/ml[7]

[1]Oda *et al.* 1997, [2]Twinner *et al.* 2001, [3]Twiner & Trick 2000, [4]Yang *et al.* 1995, [5]Kawano *et al.* 1996, [6]Kim *et al.* 1999a, [7]Zika 1984, [8]Palenik *et al.* 1987, [9]Kim *et al.* 1999b. N.D. = not determined

The mechanism and reason for ROS production in algae have not been clearly defined, and the production of $\cdot O_2^-$ and H_2O_2 are not necessarily linked. ROS are thought to be produced as antibacterial agents and to be involved in nutrient transport, oxidation or reduction of necessary or toxic metals, or the mineralisation of major nutrients such as nitrogen by the degradation of marine organic materials (Palenik *et al.* 1987). The radicals $\cdot O_2^-$ and H_2O_2 generated by *C. marina* may act as autocrine growth factors (Oda *et al.* 1995). Reactive oxygen intermediates can also cause intracellular oxidative damage when photodynamically generated through absorption of ultraviolet radiation (UVR: 320-380 nm) (Dunlap and Yamamoto, 1995). The maximum H_2O_2 production for *C. marina* (1.06 ± 0.25 μM for 14.6 x 10^4 cells, Oda *et al.* 1997) and *H. akashiwo* (0.906 ± 0.214 μM for 6 x 10^4 cells, Twiner *et al.* 2001) is far below that used in routine preventative

treatment for farmed finfish (5 mM, Arndt and Wagner 1997) and hence not considered to be a significant a toxic contributor in causing finfish mortalities (Oda *et al.* 1994; Twiner *et al.* 2001).

Irradiance has been shown to play a role in $\cdot O_2^-$ production in *Chattonella marina* with $\cdot O_2^-$ levels significantly reduced when *C. marina* cultures are dark adapted over a 24 h period (Marshall *et al.* 2001). A similar result has been found for the red tide dinoflagellate *Cochlodinium polykrikoides* by Kim *et al.* (1999a) who inferred that the generation of ROS is related to photosynthesis, with $\cdot O_2^-$ produced through auto-oxidation of an electron acceptor of photosystem I, and H_2O_2 through the superoxide dismutase (SOD) catalysed disproportionation of $\cdot O_2^-$, similar to higher plants. In contrast, it has been reported that ROS production in *C. marina* (Oda *et al.* 1998, $\cdot O_2^-$) and *H. akashiwo* (Twiner and Trick 2000, H_2O_2) is not a product of photosynthesis.

In the present work we used the herbicide DCMU [3-(3,4-dichlorophenyl)-1,1-dimethyl-urea], which blocks photosynthetic electron transfer between photosystem II and photosystem I, to test the hypothesis that $\cdot O_2^-$ production is not related to photosynthesis. We compared exponential stage cultures of *Chattonella marina* from Port Lincoln, Australia (UTCMPL01) and the Seto Inland Sea, Japan (NIES-118) to the low ROS producing green flagellate *Dunaliella tertiolecta* (CCMP-1320). Triplicate samples were treated with 1×10^{-5} M DCMU for a period of 24 h and compared to light and dark adapted treatments. The oxygen radical $\cdot O_2^-$ was measured using the luciferin analogue 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) as previously described by Lee *et al.* (1995) at a concentration of 5×10^{-6} M and standardized against 5×10^{-6} M superoxide dismutase (SOD). All results were standardised against controls consisting of GSe media (Blackburn *et al.* 1998).

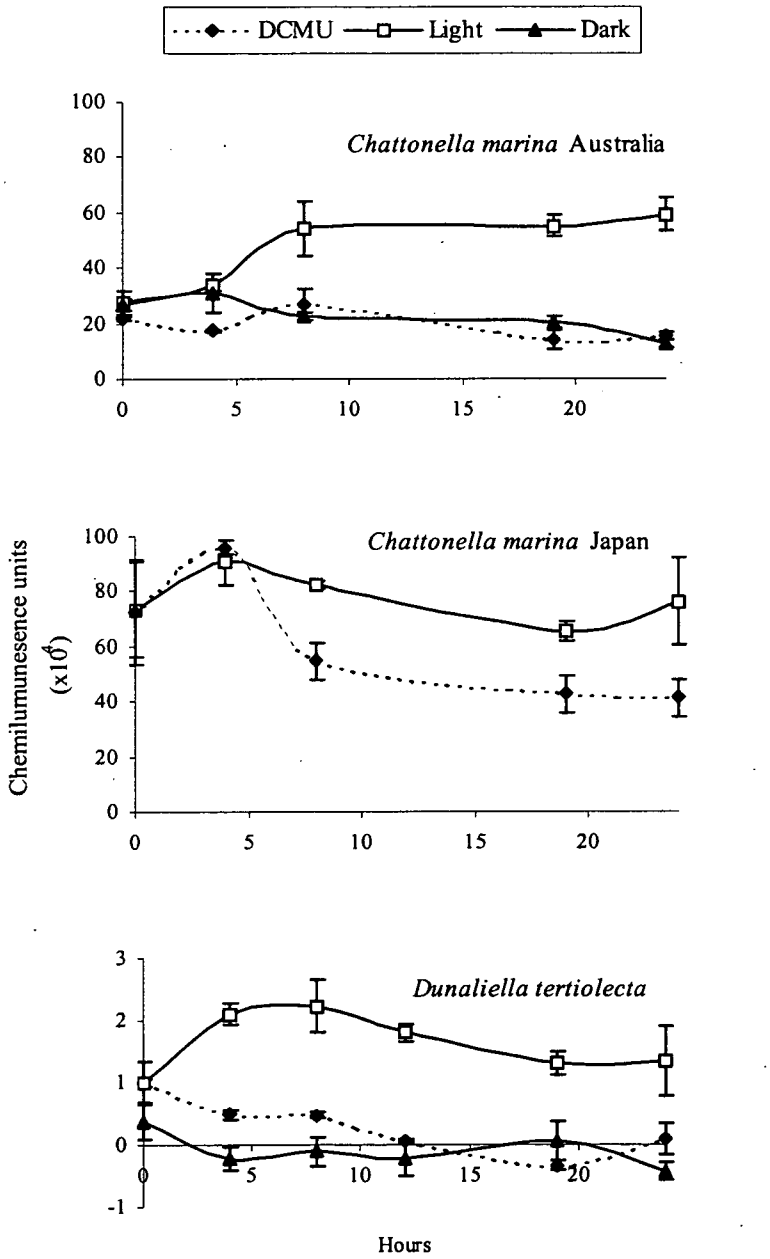

Figure 8.1. Effect of DCMU, continuous light and continuous darkness on the production of superoxide by *Chattonella marina* from Australia and Japan and the green flagellate *Dunaliella tertiolacta* over a time period of 24 hours. Note time lag of around 5 hrs before the DCMU starts to affect superoxide production for *C. marina*. Scale difference for *D. tertiolacta*..

A lag period of up to 5 hrs (Fig 8.1) was observed before the DCMU started to affect superoxide production in *C. marina*. After 8 to 24 hrs, the rate of $\cdot O_2^-$ production in the DCMU treatment did not differ significantly from the dark treatment, however both were significantly lower than in the light treatments (Table 8.2). The production of $\cdot O_2^-$ by Japanese *C. marina* was significantly greater than that of the Australian strain. . The reduction in $\cdot O_2^-$ in the DCMU treatment was not a result of decreased cell density. Examination of cells using light microscopy found no significant change in cell morphology between treatments at 24 h, however after 48 h, cell death was observed in the DCMU and dark treatment but not in the light treatment Previous DCMU experiments (Oda *et al.* 1998; Twiner & Trick, 2000), which suggested no change in ROS production in *C. marina*, may relate to the absence of adequate incubation time to allow the absorption and metabolism of DCMU.

A comparative experiment using *Dunaliela tertiolecta,* which does not produce significant amounts of $\cdot O_2^-$ when compared to GSe media controls, also resulted in a reduction of $\cdot O_2^-$ production when dark adapted or treated with DCMU. Similar to the situation in higher plants,

Table 8.2. ANOVA results showing significant differences in superoxide production between the light and DCMU/Dark treatments on Japanese and Australian strains of *C. marina* and between the Japanese and Australian strains. Differences complied when both whole culture and superoxide production per cell were considered.

Chemiluminescence per culture

Source of variation	**df**	**F ratio**	***P<***
Treatment	1	45.3	0.0001
Strain	1	106.4	0.0001
Treatment × Strain	1	11.1	0.01

Chemiluminescence per cell

Source of variation	**df**	**F ratio**	***P<***
Treatment	1	24.2	0.0012
Strain	1	58.5	0.0001
Treatment × Strain	1	12.3	0.008

photosynthesis may be a major source of $\cdot O_2^-$ radicals in all phytoplankton. The involvement of photosynthetic electron transfer in $\cdot O_2^-$ production is further supported by the rapidly induced difference between light and dark treatments, with a significant decline occurring by 4 h after *Dunaliella* (Fig. 8.1).

The reduction of $\cdot O_2^-$ production in DCMU treatments compared to light controls was similar in Australian *C. marina* (1.7 –1.8 fold) and *D. tertiolecta* (1.2-1.8 fold), but lower in the Japanese *C. marina* (0.5 fold), which may be an artifact of the high background levels of $\cdot O_2^-$ produced by the Japanese strain. The difference in $\cdot O_2^-$ production between the Australian and Japanese strain may be due to the presence of ROS quenching mycosporine-like amino acids in the Australian strain (Marshall & Hallegraeff 1999; Marshall & Newman 2002).

Photosynthesis appears to be a source of donated electrons allowing $\cdot O_2^-$ production. For higher plants under optimal conditions, minimal superoxide would be produced, as with *D. tertiolecta* which produces levels of $\cdot O_2^-$ similar to that of GSe media. The level of $\cdot O_2^-$ production by *C. marina* in the dark is one order of magnitude higher than that by *D. tertiolecta*, suggesting that *C. marina* also uses other metabolic pathways to produce $\cdot O_2^-$ besides photosynthesis.

Kawano et al. (1996) reported that the production of $\cdot O_2^-$ in *C. marina* was controlled by oxidoreductases utilizing NADPH as a source of reducing equivalents for the reduction of O_2 to $\cdot O_2^-$. The process of NADPH-dependent $\cdot O_2^-$ generation has been linked to the presence of a glycocalyx on the raphidophyte cell surface (Kim *et al.*, 2000, 2001). The reducing power to generate $\cdot O_2^-$ is most likely supported through photosynthetic electron transfer (Fig. 8.2). It is possible that photosynthesis is only indirectly linked to $\cdot O_2^-$ production through the photosynthetic reduction of $NADP^+$ to NADPH, which sustains $\cdot O_2^-$ production at the cell membrane. However it is clear that $\cdot O_2^-$ production falls dramatically within a few hours after

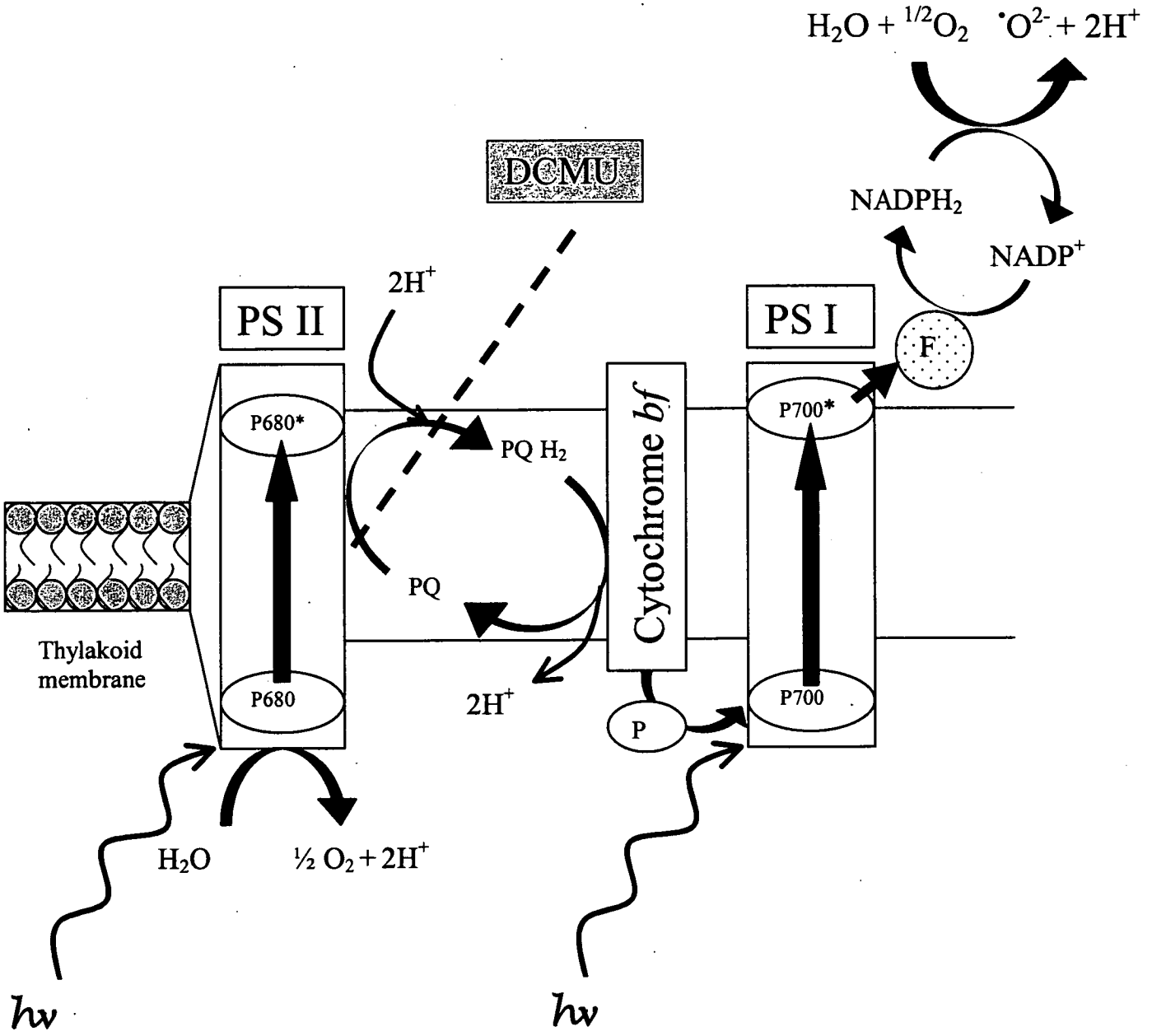

Figure 8.2. A proposed scheme for the production of superoxide through NADPH cycling linked to photosynthetic electron transfer. DCMU blocks electron transfer after photosystem II (PS II) by binding to with plastoquinone (PQ) and thus disrupting the flow of electrons. By inactivating photosynthesis at plastoquinone, ferredoxin (Fd) is not reduced and thus reduction of $NADP^+$ to NADPH via ferredoxin does not occur. Without the reduction of $NADP^+$, oxygen is not then reduced to superoxide. hv= photon energy; PC = plastocyanin; P680 = PSII reaction center chlorophyll: P700 = PSI reaction center chlorophyll.

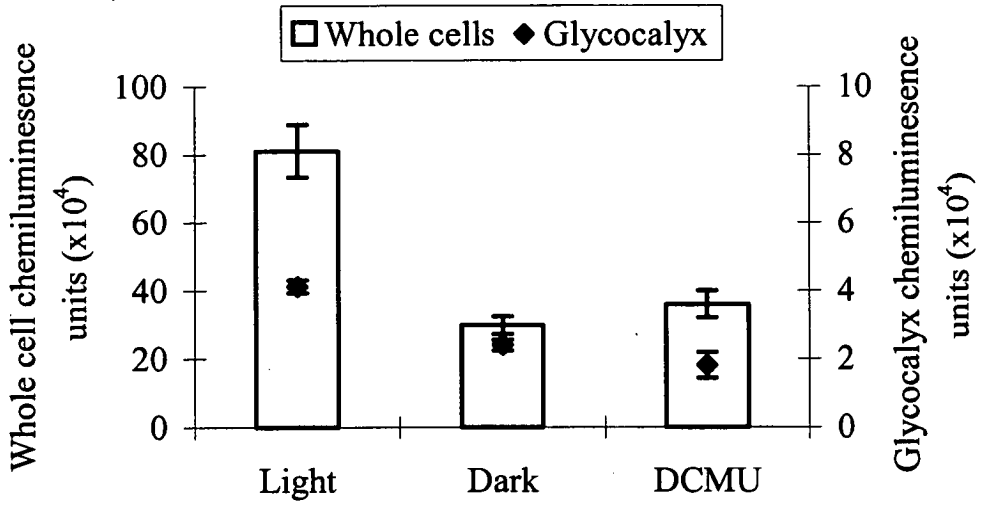

Figure 8.3. The production of superoxide by *Chattonella marina* whole cells (columns) and cell supernatant containing glycocalyx (diamonds) before and after 24 hours DCMU, continuous light and dark treatments.

the cessation of photosynthetic electron transfer. To test whether the $\cdot O_2^-$ produced by the glycocalyx was linked to photosynthesis, we measured SO produced by the glycocaylx after 24 hrs exposure to DCMU, light and dark treatments. Cells of *C. marina* were treated with DCMU as previously described, and the glycocaylx removed through gentle agitation and centrifugation at 10000 rpm for 1 min and assessed for $\cdot O_2^-$ production as previously described in the presence and absence of NADPH, as per Kim *et al.* (2000). The glycocalyx under optimal light conditions was found to produce 13-20% of the total $\cdot O_2^-$ production of whole cells in the presence of NADPH and 5-8% without NADPH. Treatment with DCMU or dark adaptation changed the rate of $\cdot O_2^-$ production in the glycocalyx proportional to that of the whole cells (Fig 8.3.). The presence of NADPH did not change this ratio. Therefore photosynthesis also has a role in $\cdot O_2^-$ production at the cell surface. Morphological examination of *C. marina* shows a close spatial association

between the chloroplasts and the cell membrane, including the glycocalyx. Thus, photosynthetically driven reduction of $NADP^+$ may contribute to the release of $\cdot O_2^-$ across the cell membrane. Oda *et al.* 1997 also inferred that NADH oxidation activity may be related to ROS generation due to the inhibition of $\cdot O_2^-$ in the presence of the iron-specific chelator Desferal. Low levels of ROS may be produced at the chloroplast membrane site as a result of reduction of $NADP^+$ to NADPH, but it is not known if this reaction alone is sufficient to produce the quantities of $\cdot O_2^-$ reported in the literature.

We conclude that the raphidophyte alga *C. marina* has an extraordinary capacity to produce $\cdot O_2^-$ partially controlled by through photosynthesis. It is not understood at a subcellular level how the *Chattonella* cells produce high levels of $\cdot O_2^-$ under apparently optimal physiological conditions. The relationship between photosynthesis and $\cdot O_2^-$ production may have important implications for the aquaculture industry in the timing of finfish mortalites, which may be reduced during darkness or shading.

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Chapter 9

Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to Damselfish (*Acanthochromis polycanthus*): The Synergistic Role of Reactive Oxygen Species and Free Fatty Acids*

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9.1 Abstract

This investigation aimed to elucidate the relative roles of putative brevetoxins, reactive oxygen species and free fatty acids as the toxic principle of the raphidophyte *Chattonella marina*, using damselfish as the bioassay. Our investigations on Australian *C. marina* demonstrated an absence or only very low concentrations of brevetoxin-like compounds by radio-receptor binding assay and liquid chromatography – mass spectroscopy techniques. *Chattonella* is unique in its ability to produce levels of reactive oxygen species 100 times higher than most other algal species. However high levels of superoxide on their own were found not to cause fish mortalities. Lipid analysis revealed this raphidophyte to contain high concentrations of the polyunsaturated fatty acid eicosapentaenoic acid (EPA; 18-23% of fatty acids), which has demonstrated toxic properties to marine organisms. Using damselfish as a model organism, we demonstrated that the free fatty acid (FFA) form of EPA produced a mortality and fish behavioural response similar to fish exposed to *C. marina* cells. This effect was not apparent when fish were exposed to other lipid fractions including a triglyceride containing fish oil, docosahexaenoate-enriched ethyl ester, or pure brevetoxin standards. The presence of superoxide together with low concentrations of EPA accelerated fish mortality rate threefold. We conclude that the enhancement of ichthyotoxicity of EPA in the presence of superoxide can account for the high *C. marina* fish killing potential.

Keywords: ichthyotoxicity, reactive oxygen species, free fatty acids, *Chattonella marina,* superoxide, eicosapentaenoic acid

9.2 Introduction

Several hypotheses have been proposed for the toxic mechanism of the fish killing raphidophyte *Chattonella marina*. The original theory put forward by Okaichi (1983) was the production of free fatty acids by *Chattonella* entering

the blood stream via the gills, resulting in fish mortalities. Subsequent studies have investigated reactive oxygen species (ROS) production and its role in gill damage (Shimada *et al.*1983, Oda *et al.* 1992). Later investigations have centred on anoxia, mucus production, respiratory, ionoregulatory and cardiovascular physiology (Ishimatsu *et al.* 1990,1991, 1997). *Chattonella marina* has also been claimed to produce a fat-soluble toxin similar in structure to brevetoxin (first characterised from the dinoflagellate *Karenia brevis:* Onoue *et al.* 1990, Kahn 1995), but the raphidophyte toxin has yet to be fully characterised chemically. The ROS superoxide ($\bullet O_2^-$), which is produced by *C. marina* at levels 100 fold higher than that of most other alga (Marshall *et al.* 2002a), has also been implicated in fish mortalities through changes in gill pathology (Ishimatsu *et al.* 1997). Ecophenotypic differences in $\bullet O_2^-$ production between Australian and Japanese strains have shown that higher $\bullet O_2^-$ production in *C. marina* correlates with faster mortality in juvenile rainbow trout (*Onchorrynchus mykiss*) (Marshall *et al.* 2001). Furthermore, $\bullet O_2^-$ is primarily controlled through photosynthesis (Marshall *et al.* 2002a). However, when superoxide levels were suppressed in dark treatments, fish mortalities did not occur. This lead to the hypothesis that a synergy between ROS and a toxic fraction could be responsible for fish mortalities (Marshall *et al.* 2001).

Chattonella marina has been found to produce high levels (10%) of free fatty acids (FFA), which have also been implicated in *Chattonella* fish toxicity (Okaichi *et al.* 1989). Both Australian and Japanese strains contain high levels of potentially toxic polyunsaturated fatty acids (PUFA) including eicosapentaenoic acid (EPA, 20:5ω3, 18-23%; Marshall *et al.* 2002b). Arzul *et al.* (1995, 1998) demonstrated that FFA including steraradonic acid (STA, 18:4ω3) and EPA from the dinoflagellate *Karenia mikimotoi* have an alleopathic effect on the diatom *Chaetoceros gracile*, and can result in haemolysis of horse red blood cells and mortality of *Pecten maximus* shellfish larvae. The present investigation aimed to elucidate the relative roles of

putative brevetoxins, reactive oxygen species and free fatty acids as the ichthyotoxic principle of *Chattonella marina*.

9.3 Methods

9.3.1 Algal Culturing

A non-axenic culture of *Chattonella marina* (UTCMPL03) isolated from Port Lincoln, South Australia, in 2002 was maintained in full strength seawater containing GSe nutrients (Blackburn *et al.* 1998) at 25°C and 150 μmol $m^{-2}s^{-1}$ light. A culture of *Dunaliella tertiolecta* (CS-175) was used as an algal control in the fish exposure experiments. Experimental cultures were kept in exponential phase through the use of semi-continuous culture techniques to ensure nutrient limitation did not occur.

9.3.2 Fish Bioassay

Damselfish (*Acanthochromis polycanthus* Bleeker) weighing 600 – 1200 mg were obtained from the University of Tasmania, School of Aquaculture, as 3 heterogeneous batches. Fish were exposed to early exponential stage cultures of *C. marina* at concentrations of 250 to 35, 000 cells ml^{-1}, which were adjusted to cell concentrations to be tested 48 h prior to fish challenge to allow equilibrium of reactive oxygen species levels. The experimental cultures were not aerated and oxygen levels were measured using a WTW Oxyguard probe. Pure FFA (EPA and STA) were obtained from Sigma Chemical Co. Other lipids used were an ethyl ester enriched 22:6ω3 (DHA) (66% of fatty acid fraction with 20% EPA) and tuna oil containing > 99.5% tryglyceride (with 25% DHA and 8% EPA). Lipids were dissolved in 2 ml of methanol and introduced to 400 ml of seawater containing 4 damselfish at concentrations of 2-25 mg l^{-1}.

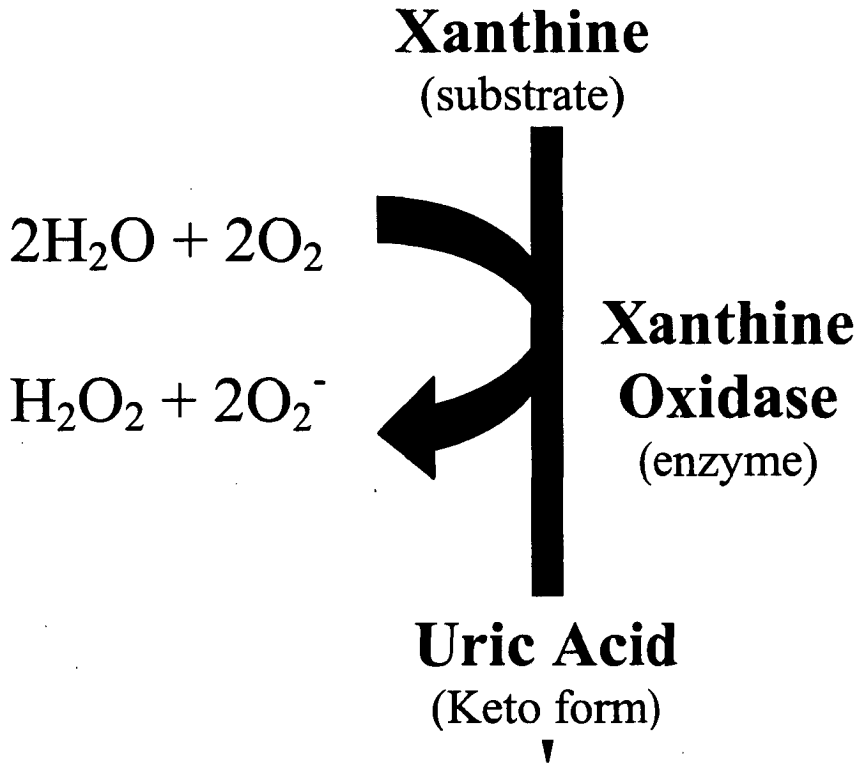


Fig.1. Xanthine catalyses oxidation of xanthine to uric acid whilst reducing O_2 to both superoxide (O_2^-) and hydrogen peroxide (H_2O_2).

9.3.3 Reactive Oxygen Species

The oxygen radical superoxide ($\cdot O_2^-$) was measured using the luciferin analogue 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) as previously described by Oda *et al.* (1992) at a concentration of 5 x 10^{-6} M and standardized against 5 x 10^{-6} M superoxide dismutase (SOD). All results were corrected for controls consisting of GSe media. Superoxide was generated though xanthine oxidase acting aerobically on a xanthine substrate resulting in the production of uric acid (Halliwell and Gutteridge 1999) as shown in Fig 1. Superoxide levels were maintained by periodically adding 5 x 10^{-6}M xanthine to 10-30 units per litre of xanthine oxidase in filtered seawater, determined to be equivalent to the production of superoxide by *C. marina* and checked by MCLA luminescence.

9.3.4 Toxin Analysis

Chattonella cells were centrifuged then extracted in dichloromethane, evaporated and partitioned between methanol and hexane to remove fats and selected pigments e.g. carotenoids. The residue for the methanol fraction was fractionated using silica flash chromatography as per Lewis *et al.* (1991) with chloroform/methanol (c:m) solutions of increasing polarity (1:0, 9:1, 7:3, 1:1, 2:8, 0:1). Fractions were assessed using a tritium labelled brevetoxin radio-labelled ligand binding (RLB) assay (Poli 1986, Hamilton *et al.* 2002) at 0.5%, 0.05%, 0.005%, 0.0005%, 0.00005% and 0.000005%. Samples were further analysed using a gradient of 2% B/min (A:0.1% formic acid (aq); B: 90% acetronitrile (aq) + 0.1% formic acid) established on an Agilent 1100 series high performance liquid chromatography system coupled to a SCIEX Qstar (Quadrupole/time-of-flight) mass spectrometer (LC/MS). The Institute of Medical and Veterinary Science, South Australia, conducted mouse bioassays using IVMS Method FH38. Duplicate mice were injected intraperitoneally with the equivalent of 3 x 10^6 cells of *Chattonella marina* from Australia and Japan (strain NIES-118), extracted in methanol and suspended in 10% Tween solution.

9.3.5 Statistical Analysis

Results were analysed using a simple regression with a significance level of 0.05.

Toxicity of brevetoxins (PbTx-2 and PbTx-9) and FFA was calculated from the survival time of the fish and expressed as fish units (FU; Ahmed *et al.* 1995). One fish unit represents the amount of toxin needed to kill a damselfish in 30 min calculated as

$$FU = (30*C)/T$$

where, C is the concentration of the toxin in mg and T is the survival time in min. Toxicity of the cells was presented as FU per 10^6 cells.

9.4. Results

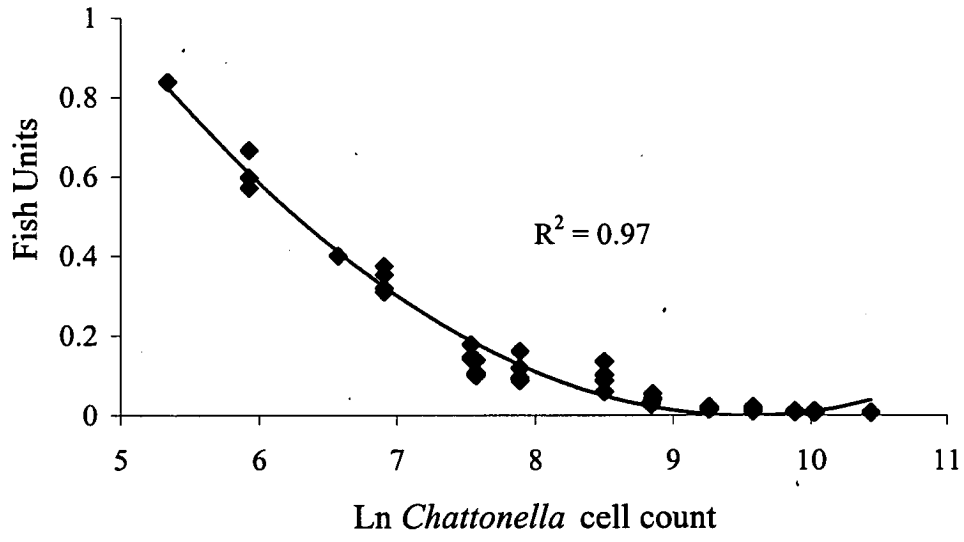


Fig. 2. Fish units as a function of cell density of *C. marina*, which was maintained under semi-continuous, culture conditions in separate 2 experiments (n=8 per treatment). Only one mortality was observed at cell densities < 1,000 cells ml^{-1} (n = 20) over a 3-h period.

9.4.1 Chattonella marina exposure and superoxide production

Damselfish exposed to non-aerated cultures of *C. marina* less than 1,000 cells ml^{-1} rarely exhibited mortality. Fish exposed to algal concentrations of 1,000 - 8,000 cells ml^{-1} suffered more rapid mortality times (89 ± 6 min) than fish exposed to cell concentrations above 8,000 cells ml^{-1} (143 ± 8 min; Fig. 2). A regression (ANOVA) model for *Chattonella* densities above 1,000 cells ml^{-1} suggests that fish mortality time is inversely correlated with cell density ($p = 0.44$) and closely correlated with superoxide levels ($p = 0.67$). Total superoxide levels were maximal in algal cultures with higher cell densities of *C. marina*, but the fastest fish mortality time correlated with low cell densities of 1,000 to 8,000 cells ml^{-1} and was associated with levels of $\bullet O_2^-$ production per cell greater than 40 chemiluminescence units (Fig. 3).

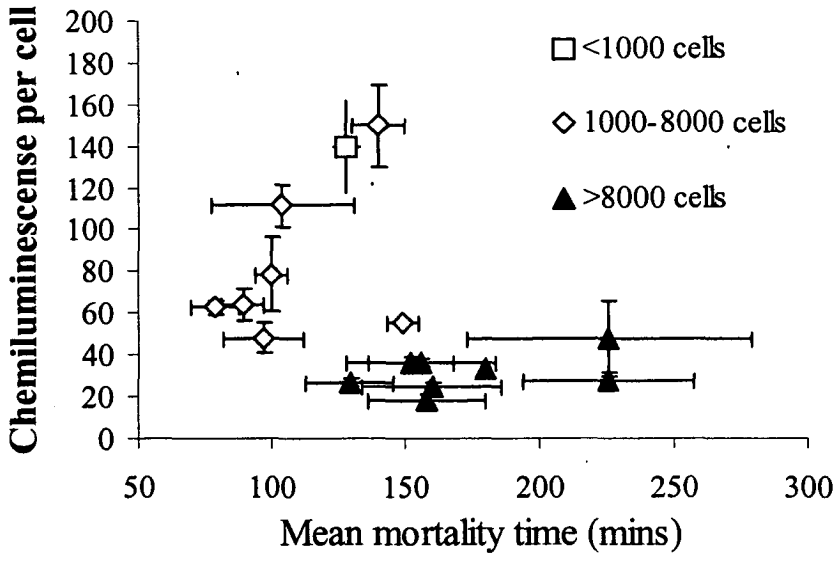


Fig. 3. Levels of superoxide per cell determined by chemiluminescence compared to mortality time of damselfish exposed to *C. marina* cultures. There is a clear distinction between low cell density (1000-8000 cells per ml) and rapid mortality time compared to high cell density (>8000 cells ml^{-1}) and longer mortality time. Only 1 mortality occurred in < 1000 cells ml^{-1} (n=20).

No fish mortalities occurred during the 6-h experiments in aerated cultures. Dissolved oxygen (DO) levels in non-aerated experiments were maintained above that of the non-aerated control by oxygen released through photosynthesis of *C. marina* cells. The higher *C. marina* density resulted in higher DO ($r^2 = 0.83$; data not shown). *Chattonella marina* in the presence of fish produced significantly higher $\bullet O_2^-$ than *C. marina* not exposed to fish, regardless of whether the cultures were aerated (Fig.4). Fish exposed to low concentrations of *C. marina* (1,000-8,000 cells ml^{-1}) rapidly developed symptoms of hyperventilation, mucus excretion, vasodilation of the gills (as seen through the opercular cover), followed by an inability to maintain position in the water column. Pre-mortality response included gulping at the water surface before sinking and lying laterally prior to respiratory cessation.

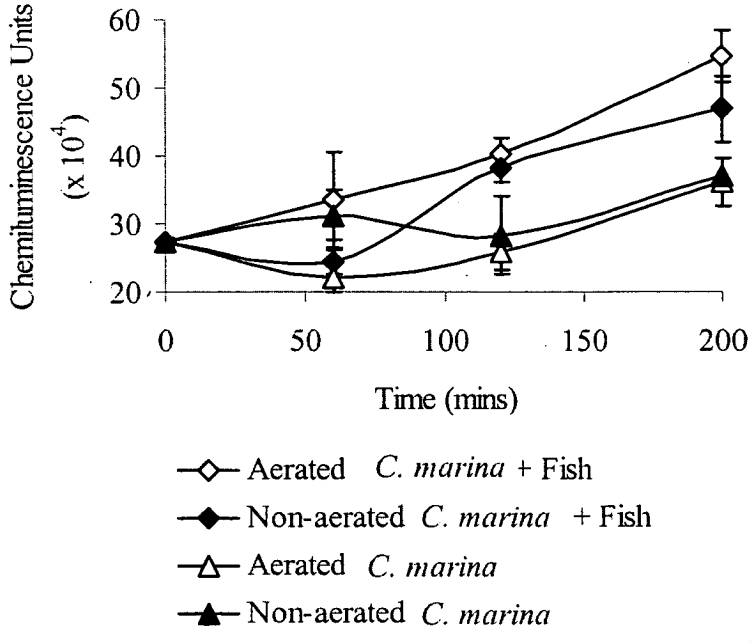


Fig. 4. The effect of aeration on total superoxide production of *C. marina* cultures as determined by chemiluminescence, in the presence and absence of damselfish. Open symbols denote aerated cultures and closed symbols denote non-aerated cultures. Diamonds denote the presence of damselfish and triangles denote no fish present.

9.4.2 Xanthine Oxidase Generated Superoxide Exposure

Superoxide was produced and maintained through periodic additions of the substrate xanthine to the enzyme xanthine oxidase for a period of 3 h. Levels of $\bullet O_2^-$ produced, observed every 15 min did not deviate significantly from those assessed in the *Chattonella* control of 6,000 cells ml^{-1} (Fig. 5). Fish exposed to the xanthine oxidase generated $\bullet O_2^-$ did not display stress behaviour for the first 150 min when compared to the filtered seawater, the non-toxic *Dunalliella* and *Chattonella* controls. After this time, the fish appeared sluggish until termination of the experiment at 180 min. No fish mortalities resulted in the xanthine oxidase control trial, nor were any behavioural responses corresponding to *Chattonella* toxicity noted.

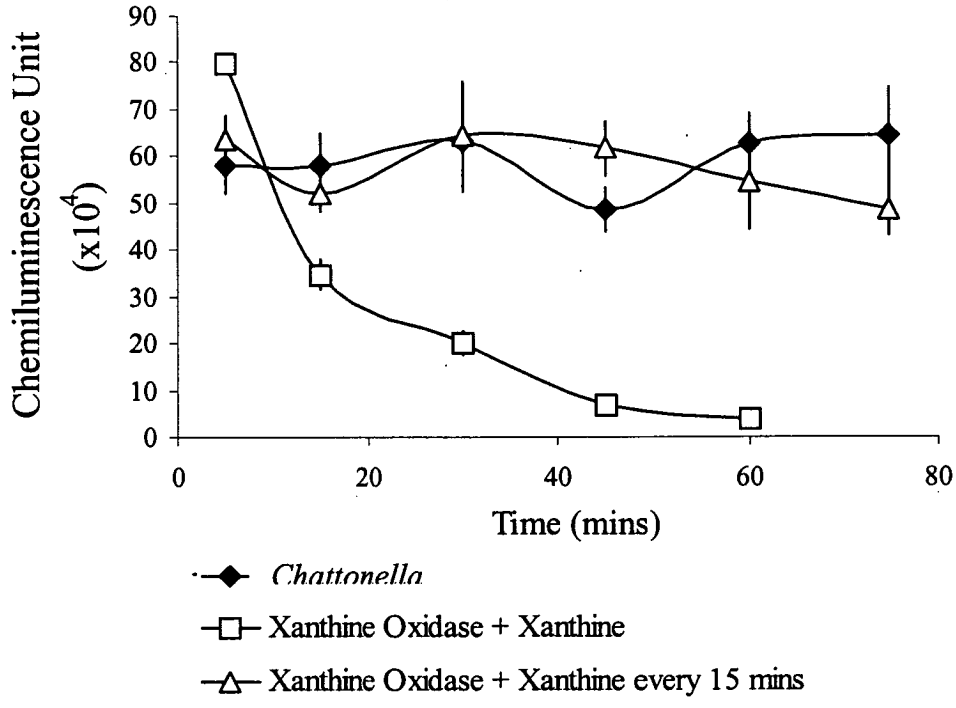


Fig. 5. Production of superoxide generated from the xanthine oxidase system compared to superoxide production by *Chattonella marina* at 6000 cells ml^{-1}. A single application of xanthine shows a gradual reduction in superoxide levels, however regular additions of xanthine maintained high levels of superoxide.

9.4.3 Brevetoxin Analysis

The RLB analysis did not indicate the presence of PbTx-like material in any of the fractions. The LC/MS experiments did not detect any of the major PbTxs (PbTx-1, -2, -3, -7, -9, -10). In the 7 $CHCl_3$:1 MeOH silica fraction we observed: (1) $[M+Na]^+$ at *m/z* 901.50, $[M+NH_4]^+$ at m/z 896.54, $[M+H]^+$ at *m/z* 897.50, and $[M+H-H_20]^+$ at *m/z* 861.50 at 29.1 min, (2) $[M+Na]^+$ at *m/z* 843.47, $[M+NH_4]^+$ at *m/z* 838.47, $[M+H]^+$ at *m/z* 821.45, and $[M+H-H_20]^+$ at *m/z* 803.45 at 27.8 min, and (3) $[M+Na\}^+$ at *m/z* 899.48, $[M+NH_4]^+$ at *m/z* 894.55, and $[M+H]^+$ at *m/z*

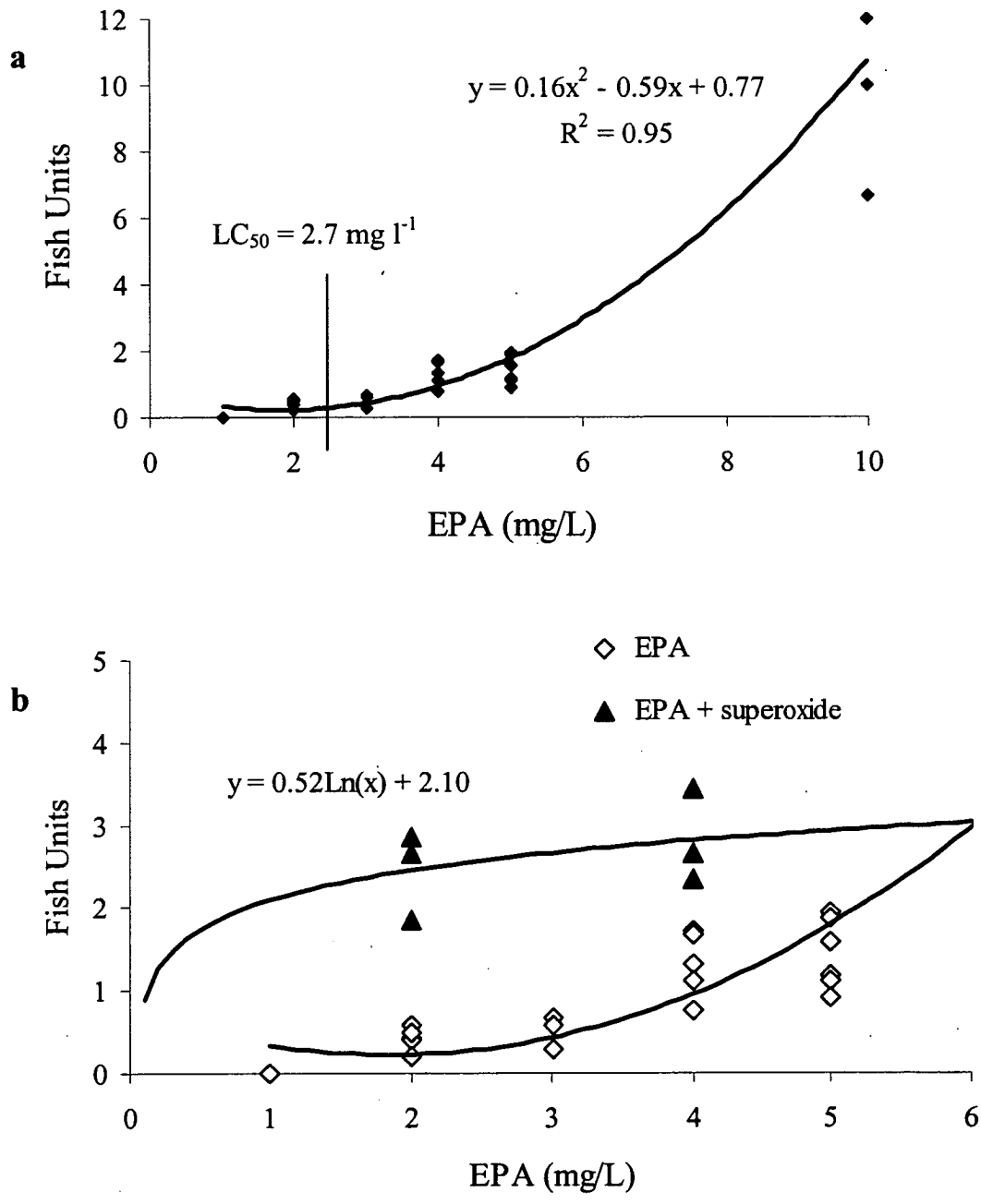


Fig. 6. Toxicity of eicosapentaenoic acid (EPA) to damselfish. (a) A fitted polynomial curve produced the response equation of $y = 0.16x^2 - 0.59x + 0.77$ ($r^2 = 0.95$). LC_{50} calculated as 2.7 mg l^{-1}. (b) Data from a combined with exposure of sublethal (2 mg l^{-1}) and lethal (4 mg l^{-1}) concentrations of the free fatty acid form of EPA in the presence of xanthine generated superoxide. The data is then fitted with a hypothetical logarithmic curve to predict the effect of low concentrations of EPA in the presence of superoxide to produce the equation $y = 0.52\text{Ln}(x) + 2.10$

877.51 at 29.6 min. These chemical species were present at very low levels and the masses do not correspond to any known PbTx's. The residual masses (0.45-0.55 Da), a function of the relative number of mass deficient elements such as oxygen present in each of the molecules, indicate that they share a very similar elemental composition to all known polyether marine toxins and perhaps are structurally similar.

Mouse bioassays on lipophilic extracts of both Australian and Japanese strains of *C. marina* did not result in mouse mortality (T = 24 h). There was no visible evidence of mouse liver abnormality or damage on *post mortem* examination. Fish exposed to pure PbTx-2 and PbTx-9 standards at 25 μg/l showed no signs of respiratory distress within the first 6 h but did display uncoordinated swimming behaviour (swimming laterally or up-side down) as described by Lewis (1992).

9.4.4 Free Fatty Acid Exposure

Exposure of damselfish to pure EPA resulted in a LC_{50} of 2.7 mg l^{-1}, with mortality of 50% of fish occurring at 155 min (Fig. 6a). Only 25% of fish died when exposed to 25 mg l^{-1} of STA, but all fish showed symptoms of being adversely impacted by a toxic substance. Lethal and sub-lethal doses of EPA and STA resulted in the fish showing pronounced opercular movement (suggesting respiratory distress), production of mucus, inactivity, an inability to maintain position in the water column and a loss of righting reflex. The gill region appeared red, suggestive of vasodilation. No symptomatic fish behaviour or mortalities occurred when the fish were challenged with the esterified fatty acids in TAG or the ethyl ester form, the latter containing 66% DHA (22:6ω3).

Fish exposed to superoxide from xanthine oxidases system in conjunction with low levels of EPA (2 and 4 mg l^{-1}) displayed similar behavioural toxic symptoms to those fish exposed to *Chattonella,* but exhibited significantly accelerated mortality rates when compared to fish exposed to EPA on its own (Table 1).

Table 1. The effect on damselfish exposed to sublethal (2 mg l^{-1}) and lethal (4 mg l^{-1}) concentrations of the free fatty acid form of eicosapentaenoic acid (EPA) in the presence and absence of superoxide, expressed as fish units (FU*). The EPA equivalent is calculated from the equation fitted to EPA exposure data shown in Fig. 4a.

EPA treatment (mg l^{-1})	EPA only *FU ± SD	EPA + Superoxide *FU ± SD	EPA equivalent (mg l^{-1})
2	0.4 ± 0.6 (n=8)	2.5 ± 0.3 (n=3)	5.6
4	1.3 ± 0.2 (n=8)	2.8 ± 0.3 (n=3)	5.9
Xanthine Oxidase	0 (n=3)	-	-

*One fish unit represents the amount of toxin needed to kill a damselfish in 30 min calculated as FU = (30*C)/T where, C is the concentration of the toxin in mg and T is the survival time in min

9.5. Discussion

9.5.1 Brevetoxin-like Compounds

Two independent investigations have failed to detect the presence of significant quantities of fat-soluble breve toxins in the Australian strain of *C. marina*. Hallegraeff *et al.* (1998) detected only trace levels of brevetoxin-like compounds in the Australian strain (0.006-0.03 fg/cell) using brevetoxin radio-receptor assays. In the present work, none of the major PbTx's were identified in an extract by either RLB or LC/MS. The RLB did not suggest the presence of any site 5 sodium channel toxin activator toxins, although it is possible that there were small amounts of lower potency toxins were present. Similarly, an intraperitoneal mouse bioassay produced negative results for neurotoxicity or liver damage for both the Australian or Japanese strains. We therefore conclude that no PbTx like compounds are detectable at significant levels in the Australian strain of *C. marina*. This is supported by the observation that exposure of damselfish to pure PTx-2 resulted in fish

behaviour comparable to that described by Lewis (1992). By contrast fish behaviour when exposed to either *C. marina* cells or the FFA - EPA and STA - was characterised by markedly different symptomology.

9.5.2 Superoxide

If *C. marina* toxicity would be solely a function of a neurotoxin, one would expect a linear increase in fish toxicity with increasing *Chattonella* cell density, rather than the inverse relationship shown by this study (Fig. 2). These results have been previously replicated for the salmonid *Onchorynchus mykiss* (Marshall, unpublished data) and support the hypothesis of a synergistic effect of ROS with some other toxic principle. Superoxide has been claimed to result in damage to the gill epithelium (Ishimatsu *et al.* 1997, Shimada *et al.* 1983), causing subsequent respiratory problems. Such toxic insult is thought to be localised at the gill membrane, where contact occurs between the gill epithelia and the *Chattonella* cells. If this were the case, the greater production of $\bullet O_2^-$ per cell should produce more fish gill lamellae damage. In the present work, cell densities of less than 1000 cells ml^{-1} did not appear to provide toxicity, possibly due to lower levels of algal cell to gill contact. Previous studies have also reported no lethality of *Chattonella* cultures at cell densities lower than 10^3 cells ml^{-1} (Haque and Onoue 2002, 2 x 10^3 cells ml^{-1}; Kahn *et al.* 1996). Temperature also affects lethal cell density for *C. marina*, with higher temperatures apparently decreasing the toxic cell density threshold (Okaichi *et al.* 1989). Superoxide production is inversely related to cell density possibly to maintain a minimum ambient environmental level of ROS as an alleleopathic protection.

9.5.3 Dissolved Oxygen

Dissolved oxygen (DO) self-evidently plays a role in the survivorship of the damselfish. Aeration provided protection against *C. marina* insult, most likely due to an alleviation of respiratory distress. Hyperventilation during periods of low DO would increase ventilation volume and velocity of *C. marina* cells crossing the gill membranes, enhancing the incidence of gill

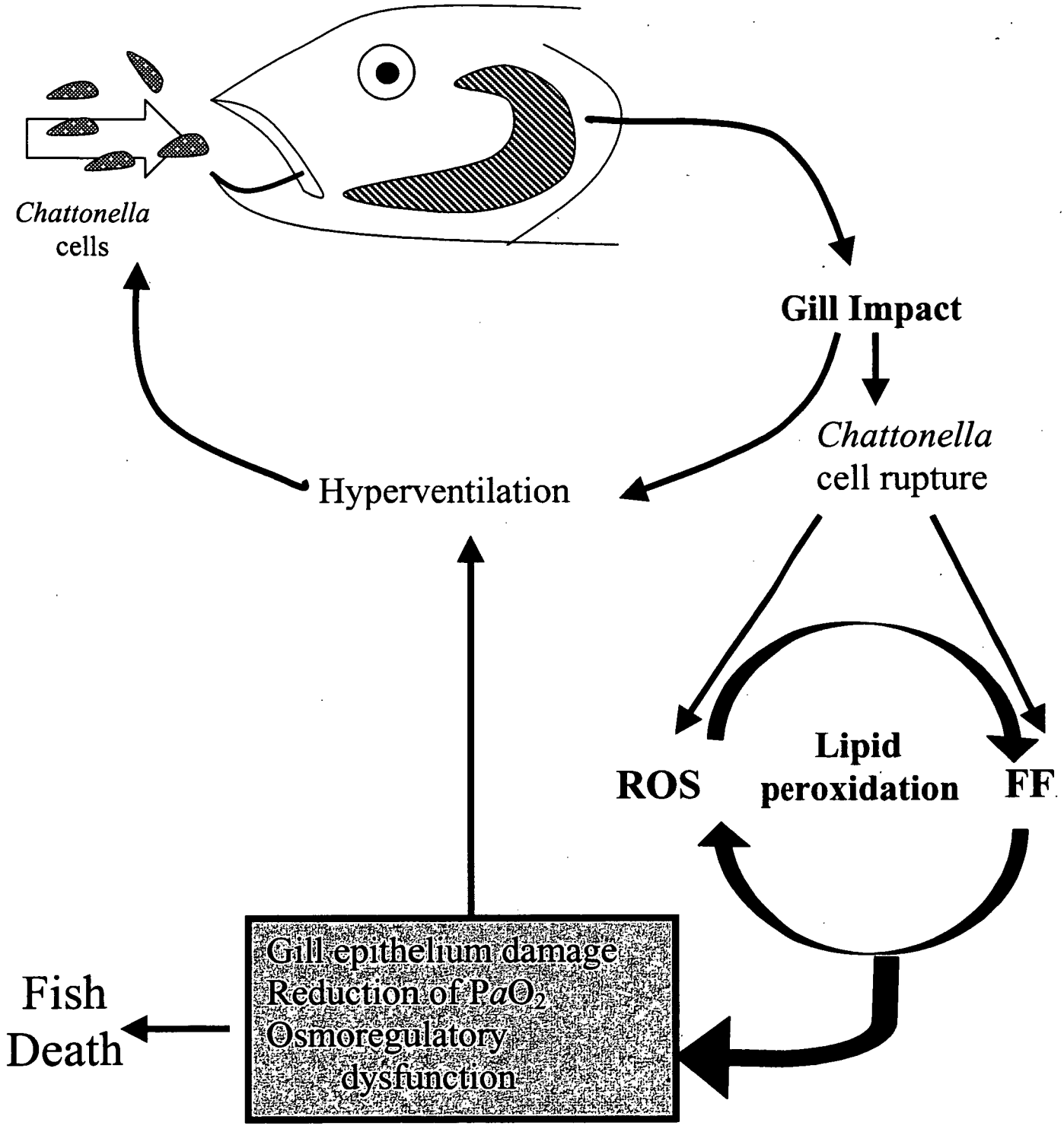
contact with *C. marina* cells. Aeration also prevented fish mortalities from the dinoflagellate *Karenia mikimotoi*, but not for *Gymnodinium cf. maguelonnense* (Jenkinson and Arzul 2001). Arzul *et al.* (1998) postulated that increased oxygen availability may be offset by greater free radical mediated cytotoxicity associated with haemolytic activity. In the present study no significant increase in $\bullet O_2^-$ production resulted from aeration of algal cultures, but significantly higher $\bullet O_2^-$ was detected in the presence of fish, indicating that the presence of fish, not aeration, stimulates the greater $\bullet O_2^-$ production by the cells, which may lead to more cytosolic activity. Lectins found in fish mucus have previously been found to stimulate $\bullet O_2^-$ production in *C. marina* (Oda *et al.* 1998).

9.5.4 Free Fatty Acids

We found that polyunsaturated fatty acids (PUFA) in the FFA form play a predominant role in *Chattonella* ichthyotoxicity. Esterified PUFA, such as TAG or an ethyl ester enrichment of DHA (both also containing EPA), are not toxic to fish. Marshall *et al.* (2002b) found that levels of EPA in *C. marina* under optimal growth conditions are 1.5-2 mg l^{-1} and that around 10% of the algal lipid is in the FFA form. By extrapolating the data for the toxicity of FFAs in the presence of $\bullet O_2^-$ using an exponential model (Fig. 6b), we postulate that small amounts of FFAs can become toxic to fish. Our model predicts that 0.2 mg l^{-1} EPA provides a fish toxicity of 1.2 fish units (FU), which is equivalent to either 4 mg l^{-1} of EPA or 1000 cells ml^{-1} of *Chattonella* culture. Both concentrations are toxic to fish.

The rupture of fragile *Chattonella* cells may lead to a rapid increase in EPA concentration in the free form in the fish buccal cavity, exacerbated by fish hyperventilation. The liberation of the free form of EPA from cell lysis has been demonstrated with epilithic diatom biofilms (Jüttner 2001). Free EPA from lysed

Fig. 7. Diagram describing possible mechanisms of fish mortality when exposed to *Chattonella* derived FFA in combination with the ROS superoxide: *Chattonella* cells are brought into contact with the fish gill lamellae through ventilation. Cell contents released from broken *Chattonella* cells hydrolyse in high ROS environment to produced higher levels of FFA and ROS. Lipid peroxidation occurs on gill and other membranes resulting in reduced respiratory and osmoregulatory capacity and allowing the transfer of FFA and O_2^- into the blood stream: Damage to the chloride cells of the gills can also lead to reduced osmoregulatory capacity. Toxic mechanisms may occur in isolation or combination.


Chattonella cells
Gill Impact
Chattonella cell rupture
Hyperventilation
ROS
Lipid peroxidation
FF
Gill epithelium damage
Reduction of PaO_2
Osmoregulatory dysfunction
Fish Death

diatom cells were shown to provide an alleleopathic role against predator grazers, with an LC_{50} for zooplankton at 34μM (= 10 mg l^{-1}). EPA has also been shown to provide an alleleopathic protection against diatoms at 1.5 mg l^{-1} (Arzul *et al.* 1998). Free fatty acids in combination with high levels of ROS may lead to the peroxidation of the biological membranes (Halliwell & Gutteridge 1999). The presence of FFAs with ROS damaged gill membranes may result in fish mortality through a number of causes; (a) breakdown of gill membranes resulting in reduced respiratory capacity, (b) absorption of FFA or superoxide into the blood stream resulting in reduced blood pH, leading to suppressed respiratory and/or osmoregularatory capacity, and/or (c) destruction of the chloride cells leading to reduced osmoregulatory capacity (Fig 7.). Fish mortality from *C. marina* cells is most likely due to a combination of all these factors. Further research is in progress to more precisely determine the minimum toxic concentrations of FFA and ROS and elucidate the respiratory and cardiovascular fish pathology associated with fatty acid toxicity.

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Chapter 10

Conclusions

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This study draws together elements of raphidophyte physiology, chemotaxonomy and ichthyotoxicity to further our understanding of how, when and where *Chattonella marina* can be a successful bloom forming species in Australian coastal waters. The physiological studies have been crucial in elucidating the role of reactive oxygen species and the chemotaxonomic study provided an important lead in testing free fatty acids for ichthyotoxic potential. This multi-disciplinary approach has been effective in answering important questions of how *C. marina* kills fish.

10.1 Chemotaxonomy

Examination of the *Chattonella* taxonomic literature (Chapter 2) lead to the suggestion that there were little morphological differences, besides cell size, between the *Chattonella* species of *C. antiqua*, *C. marina*, *C. subsalsa* and *C. minima*. Chemotaxonomic studies also suggest the existence of different ecophenotypes within the *Chattonella* species complex (Chapter 3). This study supports the combination of *C. marina*, *C. subsalsa*, and *C. antiqua* as phenotypic variants of the same species. In depth ultrastructural studies are needed to elucidate whether morphological differences warrant their designation as separate species. Lipid composition was shown to be a more reliable method of taxonomic classification for raphidophytes than carotenoid pigments, and correlated closely with recent molecular classification. Lipid composition may serve as a reliable chemotaxonomic marker in routine monitoring programs for raphidophytes.

10.2 Ecophysiology of Bloom Formation

Chattonella marina strains sourced from Japan and Australia showed similar eurythermal and euryhaline characteristics. Laboratory experiments determining conditions for maximal growth (up to 1 division /day) may not necessarily reflect the true environmental conditions for a species to bloom. In addition to temperature, salinity and light, water column stratification following storm events may play a role in triggering *Chattonella* blooms (Chapter 4). The Australian

strains have been shown to have maximum growth potential at 25°C and 30 psu salinity (Chapter 5), yet the only major Australian bloom of this species has been reported in water temperatures of 17°C despite a southern Australian distribution. There were significant differences in photophysiology between Australian and Japanese strains (Chapters 5 & 6), which could influence bloom formation at different locations. The South Australian strain of *C. marina* was adapted to non-turbid waters and high UV radiation, whereas the Japanese strain was adapted to turbid waters with low UV penetration.

10.3 Toxicology of *Chattonella*

Ecophenotypic variation may also affect the ichthyotoxic potential of the species. The Australian strain of *C. marina* produces lower levels of superoxide than the Japanese strain (Chapter 7) which may be related to the absence of the mycosporine amino acid, mycosporine-glycine (Chapter 6). There is a direct correlation with between high superoxide production and toxic potential to fish (Chapter 7). Photosynthesis contributes to the high levels of superoxide in *Chattonella* through the electron transfer system in the NADP(H) cycle (Chapter 8). The role of photosynthesis in superoxide production may result in lower fish mortalities during darkness, providing a possible mitigation strategy for finfish aquaculture.

The relationship between low *Chattonella* cell density and rapid mortality in damselfish agrees with observations of fish mortalities in aquaculture. *Chattonella marina* blooms in Port Lincoln, South Australia in 1996 were reported at 66 cells ml^{-1} (Hallegraeff *et al.* 1998) and at Esperanza Inlet, British Columbia in Sept 2002 at 50 cells ml^{-1} (Nicki Haigh, pers comm.). The toxic potential of low *C. marina* cell densities has implications for monitoring programs where cell densities are used as an alert for aquaculture. Presently there is no generally accepted management action level for *Chattonella* blooms (Anderson *et al.* 2001). Determination of this action level should take into account that the incidence of high cell densities for *C. marina* may not always coincide with maximum potential risk in terms of fish kills.

The absence of brevetoxins in the Australian strain also has implications for bloom management. In Australia or overseas there have been no reports of fish flesh contaminated with brevetoxins after a raphidophyte bloom (Anderson *et al.* 2001). If raphidophytes are universally found not to produce brevetoxin-like compounds, it may be acceptable to harvest fish prior to and during raphidophyte blooms as an emergency mitigation strategy for finfish aquaculture.

High levels of the toxic fatty acid eicosapentaenoic acid (EPA) in *Chattonella* have been shown to constitute an alternative toxic principle to the hypothesis of neurotoxins (Chapter 9). The presence of superoxide enhances the ichthyotoxicity of the free fatty acid form of EPA and can account for the high *C. marina* fish killing potential. The principle of toxic fatty acids is supported by previous work of researchers investigating lipid toxins in fragile gymnodinioid algae (Arzul *et al.* 1995,1998, Jenkinson & Arzul 2001, Okaichi 1983, Okaichi *et al.* 1989). Inter- and intracellular hydrolysis of fatty acids is widely accepted as the cause of a variety of toxic syndromes (Halliwell & Gutteridge 1999). The precise mechanism by which free fatty acids are causing fish mortalities needs to be addressed.

This study has contributed to our knowledge of bloom formation and the toxic principle of *Chattonella* by showing:

- There exists variation in toxicity between ecophenotypes of *Chattonella*, most likely related to environmental physiology.
- Breve toxins may not play a role in *Chattonella* ichthyotoxicity, at least not in the Australian strain
- Superoxide alone does not cause fish mortality

- High levels of free fatty acids, especially EPA, found in *Chattonella* are confirmed ichthyotoxins
- A synergy between reactive oxygen species and free fatty acids increases the toxic potential of *Chattonella*.

Results of this work contribute towards our ability to predict *Chattonella* blooms and their toxic potential, as well as providing a background from which mitigation strategies can be developed for the finfish aquaculture industry. The relationship between ROS, fatty acids and cellular damage has important implications also for studies of the toxic mechanisms of selected dinoflagellates, as well as other disciplines such as food technology and medical research.

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